MANOMETRIC TECHNIQUES and TISSUE METABOLISM

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INTRODUCTION TO THE SECOND EDITION

The experience gained during the past few years and the rather gratifying acceptance of the first edition have prompted us to revise and rewrite portions of this volume. In doing this we have attempted to correct errors appearing in the original edition, to add new and valuable techniques which have been developed or with which we have become familiar recently, and to omit certain specialized sections which were rarely used. These changes have not altered the size of the book appreciably, but the content is significantly different from that of the first edition; we hope the alterations will improve its usefulness.

The objectives and the principles are still the same--we regard this as an attempt to place in the hands of the beginner sound basic methods which will aid his work. This is a practical manual to be used in the laboratory rather than a reference volume to be kept on the shelf. Therefore, the book has been retained in its original form with only minor modifications designed to save space; the substitution of a heavier cover should improve its durability. It is anticipated that the volume will be revised whenever warranted.

We are indebted to all of the contributors for their cooperation, for only through a cooperative effort has the publication of this volume been possible. Permission to reproduce certain of the figures has been granted by Arthur H. Thomas (Figs. 45, 46), Central Scientific Company (Fig. 47), G. M. E. (Fig. 18), and by E. Machlett and Son (Figs. 13, 16, 28, 30, 34).

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Chapter I

THE WARBURG CONSTANT VOLUME RESPIROMETER

INTRODUCTION

Manometric methods for estimating exchange of gases have been in use in the study of both chemical and biological reactions for generations. A wide variety of techniques have been employed and many types of apparatus have been developed. The type of respirometer which has met with widest use is almost universally known as the "Warburg" instrument, although, as pointed out by Warburg (1926), it has a long history. In essence the present instrument is a modification of a "blood-gas manometer" described by Barcroft and Haldane (1902) or of that described by Brodie (1910). The essential principle involved is that at constant temperature and constant gas volume any changes in the amount of a gas can be measured by changes in its pressure. This method is most commonly applied to measurements of oxygen uptake. We shall therefore first describe its principles in terms of oxygen uptake and later consider other uses to which the instrument may be applied.

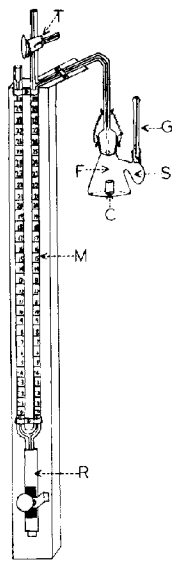

FIG. 1

The Warburg constant volume respirometer.

APPARATUS

F = flask
S = sidearm
G = sidearm stopper with gas vent
C = center well (for alkali)
M = manometer proper
R = fluid reservoir which, by adjustment of the screw clamp, serves to alter the level of the fluid in the manometer
T = three-way stopcock

The scale of the manometer is graduated in centimeters (numbered) and in millimeters. Normally one records readings in terms of millimeters.

The apparatus (Fig. 1) consists of a flask (F) (detachable) sometimes equipped with one or more sidearms (S), attached to a manometer (M) containing a liquid of known density. The flask is immersed in a water bath at a constant temperature, and between readings the system is shaken or whirled to promote a rapid gas exchange between the fluid and the gas phase. It is assumed that the temperature of the manometer, which is not immersed, does not differ greatly from that of the flask. Details of the apparatus have been described by Burk and Milner (1932), Dixon (1943), Perkins (1943), Warburg (1923, 1924, 1926) and others. Further details of shaking apparatus will be found in Chapter 5.

The manometer has (as shown in Fig. 1) an open and a closed end. A given point on the closed side of the manometer (usually 150 or 250 mm.) is chosen, and the liquid in the closed arm of the manometer is always adjusted to this point before recording pressure changes.

GENERAL PRINCIPLES

Suppose that one has an oxygen consuming reaction going on in the flask. One adjusts the closed side of the manometer (with stopcock open) to 250 mm. (by adjusting the screw clamp on the fluid reservoir of the manometer), closes the stopcock, and reads the open

side of the manometer. Assume that the level of the liquid in this arm is 249 mm. (Fig. 2). This reading of 249 is recorded. After 10 minutes time the liquid has gone up in the closed arm and down in the open arm. One again adjusts the closed arm to 250 mm. and thus holds the volume of gas in the flask constant. The reading on the open arm is now 220 mm. For both the initial and 10 minute readings the fluid in the closed arm of the manometer was adjusted to 250 mm., but during the interval the reading on the open arm dropped from 249 to 220 mm. (29 mm.) as a result of oxygen consumption in the flask. If one knows the gas volume of the flask (V_g), the volume of fluid in the flask (V_f), the temperature of operation, the gas being exchanged and the density of the fluid in the manometer, it is possible to calculate the amount of gas used up (or given off), providing only one gas is being changed. There are methods for handling alterations in the amount of more than one gas; these will be described later. The essence of the method is to hold the gas and fluid volumes constant and to measure the decrease or increase in pressure when one gas alters in amount.

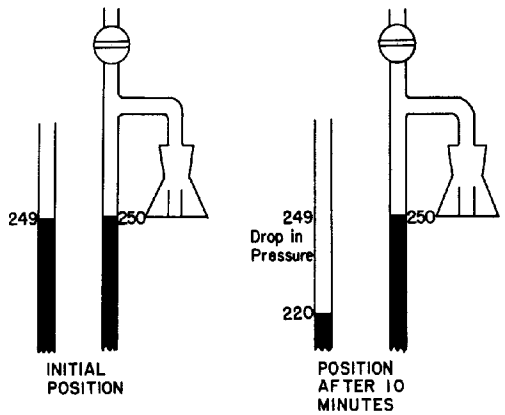

FIG. 2

Diagram illustrating the determination of pressure change.

DERIVATION AND MEANING OF FLASK CONSTANT

Fundamentally this consists of so calibrating the system that from the observed pressure changes one can calculate the amount (in $mm.^3$ or micro liters (μl) at 0°C. and 760 mm. pressure) of gas utilized or given off.

The following symbols are employed:

Let h = the observed change in the manometer (open side) reading in mm.
x = μl. gas (0°C., 760 mm. pressure)
V_g = Volume of gas phase in flask including connecting tubes down to the "zero" point (150 or 250 mm. on closed end of manometer).
V_f = Volume of fluid in vessel.
P = Initial pressure in vessel of the gas involved in the determination. This is actually the partial pressure of the particular gas in a gas mixture. If this gas mixture contains water vapor, the partial pressure of the gas involved in the determination will be less than its partial pressure in the dry condition. Hence if P is defined as dry gas, P - R should be used in equations involving moist gases.
P_o = 760 mm. Hg (standard pressure) expressed in terms of the manometer fluid:

$$P_o = 760 \times 13.60 \text{ (where 13.60 is the specific gravity of mercury)} / \text{Specific gravity of manometer fluid.}$$

T = Temperature of bath in absolute degrees (= 273 + temp. in °C.).
α = Solubility in liquid in vessel of gas involved (expressed as ml. gas/ml. liquid when gas is at a pressure of one atmosphere (760 mm. Hg) at the temperature T).
R = Vapor pressure of water (or other fluid) at temperature T. Inside the flask one has both fluid and gas. This fluid will exert a vapor pressure (R) in the gas phase and some gas will dissolve in the fluid.

In the gas phase one has gas (V_g) at a temperature (T) and at a pressure P - R (P - R = partial pressure of gas involved less the vapor pressure or the fluid). One can change this gas volume to standard conditions using this formula:

$$PV/T = P'V'/T'$$

(let prime symbols be standard conditions, i.e. V' = gas volume standard conditions, P' = P_o = 760 mm. Hg, T' = 273 = 0°C.). Hence in the flask:

$$(P - R)\ V_g/T = P_o\ V'/273$$

$$\text{and gas at standard conditions} = V' = \frac{V_g \frac{273}{T} (P-R)}{P_o}$$

Some gas is dissolved in the fluid initially. The amount of gas in the fluid is:

$$V_f \alpha\ (P - R)/(P_o)$$

Where α is the solubility of the gas (in ml. gas/ml. fluid) at a partial pressure of one atmosphere. The $(P - R)/P_o$ converts the solubility at one atmosphere to that actually existing in the flask.

This relationship holds, as Henry's law states, "The concentration of dissolved gas is directly proportional to the concentration (pressure) above the fluid." Hence if α is the solubility at P_o (one atmosphere) the solubility at the actual pressure existing in the flask, P - R (atmospheric pressure less than that due to water vapor), will be $\frac{P - R}{P_o}$. Virtually nothing is known of the relationship between chemical structure and solubility of gases so that one has to determine the solubility empirically. There is thus a different solubility for each gas in each solution. It is known, however, that the solubility of individual gases in a mixture is almost independent of the pressure of other gases, i.e., the solubility of oxygen at a given pressure and at a given temperature will be the same whether N_2, CO_2 or other gases are present or not.

From the considerations above, the gas present at the start was that in the gas phase plus that in the fluid phase or:

$$\text{Gas at start} = \underbrace{V_g \frac{273}{T} \frac{(P - R)}{P_o}}_{\text{Gas phase}} + \underbrace{V_f\ \alpha\ \frac{(P - R)}{P_o}}_{\text{Fluid phase}}$$

At the end of the observation period this gas has been changed by the amount x which has resulted in a pressure change of h mm. If gas is taken up, h is negative; if gas is given off, h is positive. We will here assume that it is taken up. The pressure is now (P - R - h) rather than the initial value (P - R).

Gas phase is thus: $V_g \frac{273}{T} \frac{(P - R - h)}{P_o}$

Liquid phase: $V_f\ \alpha\ \frac{(P - R - h)}{P_o}$

$$\text{Gas at end} = V_g \frac{273}{T} \frac{(P - R - h)}{P_o} + V_f\ \alpha\ \frac{(P - R - h)}{P_o}$$

Gas taken up (x) is that which was present initially less that which appears at the end.

x = initial gas - final gas

$$x = \left[V_g \frac{273}{T} \frac{(P - R)}{P_o} + V_f \alpha \frac{(P - R)}{P_o}\right] - \left[V_g \frac{273}{T} \frac{(P - R - h)}{P_o} + V_f \alpha \frac{P - R - h}{P_o}\right]$$

$$= V_g \frac{273}{T} \frac{(P - R)}{P_o} + V_f \alpha \frac{(P - R)}{P_o} - V_g \frac{273}{T} \frac{(P - R - h)}{P_o} - V_f \alpha \frac{P - R - h}{P_o}$$

$$= V_g \frac{273}{T} \frac{h}{P_o} + V_f \alpha \frac{h}{P_o}$$

$$x = h \left[\frac{V_g \frac{273}{T} + V_f \alpha}{P_o}\right] = h\,k$$

Note that V_g, T, α, V_f and P_o are known and, for a given experiment, are constant; these values determine the flask constant k with which one can convert mm. pressure change into µl. O_2 taken up.

Summary:

x = amount of gas exchanged = h (alteration in reading on open arm of manometer) k (flask constant)

$$k = \text{flask constant} = \frac{V_g \frac{273}{T} + V_f \alpha}{P_o}$$

Example: A Warburg flask has a total volume of 12.616 ml. up to the 250 mm. mark on the manometer. To measure oxygen uptake in this flask by yeast at 28°C., we add 1 ml. of yeast suspension, 1 ml. of 0.1 M glucose, 1 ml. of M/50 phosphate buffer. In the center cup of the flask we place 0.2 ml. 10% KOH to absorb the carbon dioxide the yeast may produce. What flask constant should be employed?

V_f = 3.2 ml. = 3,200 µl.

V_g = total volume - fluid volume = 12.616 ml. - 3.2 ml. = 9.416 ml. = 9,416 µl.

T = 273 + 28 = 301 $\qquad \alpha = 0.027 \qquad P_o = 10{,}000$

$$k_{O_2} = \frac{V_g \frac{273}{T} + V_f \alpha}{P_o} = \frac{9{,}416 \times \frac{273}{301} + 3{,}200 \times 0.027}{10{,}000} = \frac{8540 + 86.0}{10{,}000} = 0.863$$

Difference in pressure in mm. times 0.863 = µl. gas.

(Chapter Six describes methods of determining the flask constants.)

Two points may be confusing. One, the choice of 0.027 for α, will be explained in the next section. The other is the use of P_o = 10,000. The manometer in this case was filled with "Brodie's Solution" composed as follows:

23 gram NaCl
5 gram Sodium Choleate (Merck)
in 500 ml. water
Usually colored with dye, e.g. Evan blue or acid fuchsin.

This has a density of 1.033 so that

$$P_o = 760 \times \frac{13.60}{1.033} = 10,000$$

THE SOLUBILITY OF OXYGEN

The solubility of oxygen is expressed in Table I as ml. O_2/ml. fluid when the gas is at one atmosphere pressure. This term, α, is sometimes referred to as the "Bunsen Coefficient".

The α value of a particular gas is influenced by two factors. First, as is apparent from the values in Table I, the solubility of gases decreases as the temperature rises. Second, the solubility of gases is appreciably diminished by the presence of dissolved solids (or liquids, but not gases) in the fluid. This is thought to be due to the hydration ("solvation") of the solute, which leaves less free solvent available for dissolving the gas. These effects are shown in Table II constructed from data in the International Critical Tables, Volume III, p. 271 (1928). Tables on the solubility of gases other than oxygen will be found in Chapter 6.

TABLE I

The Solubility of Oxygen

Data as ml. gas dissolved per ml. fluid
when gas is at 1 atmosphere pressure (α value)

Temperature °C.	Ringer's Solution	Water
0		0.04872
10	0.0480	0.03793
15	0.0340	0.03441
20	0.0310	0.03091
25	0.0285	0.02822
30	0.0260	0.02612
35	0.0245	
40	0.0230	
	Dixon (1943)	International Critical Tables (1928)

TABLE II

The Influence of Salts Upon the Solubility of Oxygen

Data in terms of ml. O_2 dissolved per ml. solution (α value)

	HCl, 15°C	HCl, 25°C	1/2 H_2SO_4, 25°C	NaCl, 25°C
Conc.				
0.0 M	0.034	0.028	0.028	0.028
0.5 M	0.033	0.027	0.027	0.024
1.0 M	0.031	0.026	0.025	0.020
2.0 M	0.028	0.025	0.023	0.016

Although the effect of salts on oxygen solubility appears large, it actually has little effect on flask constants for oxygen uptake. For example, a change from pure water to 2 M NaCl changes the α value from 0.028 to 0.016. This lowers the flask constant by $\frac{0.012 \times Vf}{10,000}$. In the case described above, the $\underline{k}$ instead of being 0.863 would be, with 2 M NaCl, 0.859.

THE THERMOBAROMETER

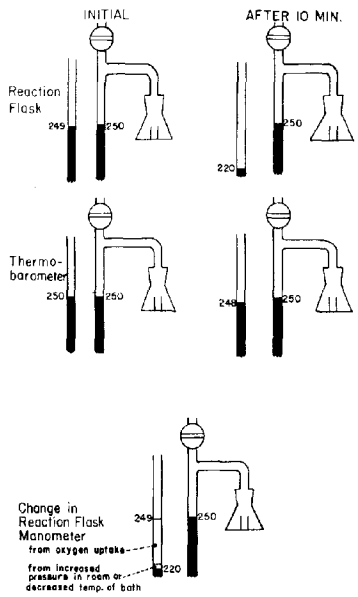

FIG. 3

Diagrams illustrating the use of the thermobarometer.

In the development of the flask constant, k, a value P was employed which was assumed to remain constant from the beginning to the end of a given period. This "P" represented the initial atmospheric pressure. The pressure in the room and the temperature of the water bath are likely to change, however, and these changes are corrected for by a thermobarometer. The thermobarometer consists merely of a Warburg manometer with a flask containing water attached; the volume of water is not critical.

Refer first to Fig. 3, initial, which represents the first reading; the reaction flask reads 249, the thermobarometer 250. At the end of a given period the reading on the reaction flask has dropped to 220, i.e., 29 mm. During the same time changes in the temperature of the bath or increased pressure in the room have caused the reading on the thermobarometer to drop to 248, i.e., 2 mm. The decrease in pressure observed in the reaction flask was due to two things: the use of some of the oxygen in the gas space inside the flask (27 mm.) and the external changes of temperature and pressure (2 mm.). Correction of the readings obtained for changes registered by the thermobarometer are obvious after a brief study of Table III which illustrates actual experimental data.

If the level of liquid in the open arm of the thermobarometer has risen there has been a decrease in pressure in the room or an increase in temperature of the bath. For reaction flasks which have registered a drop in pressure the observed decrease is smaller than the actual decrease by the amount the thermobarometer fluid has risen; hence the rise in the thermobarometer reading is added to the observed pressure drop. If the reaction flasks have registered a rise in pressure, the increase in the thermobarometer reading is subtracted from this observed rise. Examples to illustrate these points are given in Tables III and IV.

TABLE III

Corrections for Thermobarometer Changes

			Method 1			Method 2			
Time	Thermobarometer		Respiring Flask #1			Respiring Flask #1			
		Change (total)	Reading	Change	True Change	Reading	Chg. Interval	True Chg. Interval	Sum
0	250 mm.		249			249			
60	257 mm. (+7)	+7	248	-1	-8	248	-1	-8	-8
120 min.	259 mm. (+2)	+9	243	-6	-15	243	-5	-7	-15
0	250		249						
60	236	-14	111	-138	-124				

TEMPERATURE CONTROL

How accurately must the temperature of the bath be controlled? Two situations must be considered:

I. The whole bath is at a constant temperature but has dropped 1° below that accepted for calculation. The thermobarometer has responded to this drop in temperature so the main error involved arises from using the wrong flask constant. Suppose that in the case described in Table IV the temperature throughout the bath dropped to 27° C. and the data were calculated for 28° C. At 28° the k_{O_2} is 0.942; at 27° it is 0.945. If the measurements are actually made at 27° C., but the factor for 28° C. is used, an error of about 0.3% is introduced.

II. If the bath is not uniform in temperature a flask at a point 1° C. higher in temperature than another flask would indicate a pressure corresponding to about 33 µl. of gas more per 10 ml. of gas volume (difference of 0.05° C. = 1.7 µl.).

Hence two factors are important; first, that the temperature be held at the point desired, and second and more important, that the temperature of the entire bath be held uniform to within 0.05° C. This latter factor necessitates vigorous stirring of the water in the bath.

SAMPLE CALCULATIONS

The data of Table IV illustrate the method of calculating the µl. oxygen uptake from the observed changes in the level of the manometer fluid of the thermobarometer and of the reaction flask manometer. Two methods of calculation follow:

Total uptake method:

The application of this method is illustrated in columns 4, 5, 6 and 7 of Table IV. The uptake in mm. is calculated by subtracting the initial reading (246) from all subsequent readings (column 4). The thermobarometer correction is obtained by subtracting the initial reading (265) from all subsequent readings (column 5). Since in the interval from 10^{55} to 11^{00} the total uptake of 19 mm. in the reaction flask was due in part (1 mm.) to thermal or barometric changes, the real uptake was 19 - 1 = 18 mm. (column 6). This value times the flask constant for the conditions employed gives the µl. oxygen taken up (column 7).

TABLE IV

Calculating Oxygen Uptake from Manometer Readings

Time	Reading, Thermobarometer	Reading, flask	Total method				Interval method				
			Change, in mm.	Thermobarometer correction	Actual change, in mm.	O_2 uptake	Change, in mm.	Thermobarometer correction	Actual change, in mm.	µl. O_2 uptake	Sum
	mm.	mm.									
10^{55}	265	246	-	-	-	-	-	-	-	-	-
11^{00}	264	227	-19	-1	-18	17.0	-19	-1	-18	17.0	17.0
11^{05}	264	194	-52	-1	-51	48.1	-33	0	-33	31.1	48.1
11^{10}	264	159	-87	-1	-86	81.0	-35	0	-35	32.9	81.0
11^{15}	264	122	-124	-1	-123	115.9	-37	0	-37	34.9	115.9
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)

Flask: 1 ml. yeast suspension, 1 ml. M/200 KH_2PO_4, pH 4.5, 0.5 ml. water, 0.5 ml. 0.032 M glucose; glucose in sidearm, tipped in at 10^{55}.

Volume flask = 13.5 ml.; k_{O_2} = 0.942 Temp. 28° C.; 0.2 ml. KOH in center cup.

Interval uptake method:

This method is applied in the calculations shown in columns 8 to 12 of Table IV. Each reading is subtracted from the one following it (i.e., 246 from 227; 227 from 194, etc.) giving the change (column 8) over the interval. A similar calculation is made for the thermobarometer (column 9) from which the actual change (column 10) is readily apparent. These interval values are multiplied by the flask constant (column 11) to yield the uptake per interval and are added to yield the total uptake (column 12).

Although this method appears more laborious, it offers some advantages, especially when the rate of oxygen uptake is changing. In this case, for instance, the uptake during the first five minutes (17.0 µl.) is not the same as in the succeeding 5 minute intervals, for there is a tendency for the rate to increase throughout the determinations. This increase is not readily apparent when calculated by the "total method" and may even be overlooked in graphing.

Many laboratories have found it convenient to employ mimeographed or printed tabular forms for recording manometric data. The data may be recorded there permanently or temporarily before transfer of pertinent information to a permanent notebook. Two examples of such data sheets are shown in Fig. 4. The upper section is a reproduction of a form which is printed on the back of $8\frac{1}{2}$ x 11 inch graph paper and spirally bound into a notebook. Data are recorded and calculated on the printed sheet, and the results are plotted on the opposite sheet of graph paper. The readings for any one flask are recorded horizontally across the page. In the lower portion of Fig. 4 is shown a mimeographed sheet on which data for each flask are recorded in vertical columns.

It is convenient to record flask constants on a card and to secure the card inside the front or back cover of the notebook by means of a Scotch tape hinge. The card can then be flipped over, so that it projects beyond the cover of the book, where it can be referred to readily during the calculation of results.

THE USE OF THE WARBURG INSTRUMENT FOR THE MEASUREMENT OF RESPIRATION OF LIVING CELLS

Physiologically there are two meanings for the word "respiration". The older meaning confines the term to the actual uptake of gaseous oxygen. It was later realized that oxidations could occur (by the removal of hydrogen or electrons) without employing gaseous oxygen and so the term respiration was broadened to include any reaction by which the cell obtained energy, whether or not it involved gaseous oxygen as such. This has resulted in some confusion since the meaning of the term thus differs with various groups of investigators. For the purposes of this outline the following definitions are employed:

Respiration: The uptake of gaseous oxygen.
Fermentation: The transformations which occur in living cells (or enzymes therefrom) which do not employ gaseous oxygen.

In the case of most cells, as contrasted to many enzyme preparations, the utilization of oxygen results in a release of carbon dioxide. If these two gases (CO_2, O_2) are the only ones involved, one can measure the respiration (O_2 uptake) by absorbing the liberated carbon dioxide in alkali. In the presence of alkali the carbon dioxide pressure in the air is zero within the limits of measurement. The gas exchange caused by the respiration is oxygen absorption plus carbon dioxide liberation. But the alkali keeps the carbon dioxide pressure zero, hence the change noted on the barometer is due solely to the oxygen utilization. The excess of carbon dioxide in solution, of course, continually distills over into the alkali, but it does not affect the observed pressure changes.

THE ABSORPTION OF OXYGEN

The absorption of oxygen by the respiring tissue takes place almost entirely from the oxygen in solution. This is the principle reason for shaking the fluids in the respiro-

Title: Run No.:

Date: Temp.: Contents of inner well: Gas phase:

No.	Flask Contents	Time; Reading in mm.; Change in mm.; Gas change in c. mm.
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		

Signed

Prep: pH: Temp: Date: Noteb: Page:

Flask	T.B.																												
In cup																													
Side I																													
"2/well			/				/				/				/				/				/				/		
K_____																													
Time	Int.	A	△	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
1047 IM																															

FIG. 4

Forms for recording manometric data.

meter, i.e. to obtain a fluid phase saturated with the gas phase. But one must, under practical circumstances, take care that the rate of oxygen uptake by the tissue is not greater than can be replaced by the diffusion of oxygen from the atmosphere into the fluid. If the rate of oxygen uptake is so high that the oxygen is used up faster than it can diffuse into the liquid, then the rate of respiration observed is dependent upon the rate at which oxygen diffuses into the fluid and has little to do with the potential rate of the reaction itself.

The rate at which gas diffuses into a liquid is dependent upon the surface layer of the liquid. The gas may be thought of as moving across a film of surface, and the theory of such diffusion has been well worked out. Roughton (1941) has described methods for correcting for diffusion errors when they exist. However, for virtually all respiratory measurements it is sufficient to note that by shaking the flasks a continual new surface is exposed to the gas by virtue of the turbulence of the fluid in the flask. Hence, the greater the rate of shaking, the greater the rate of diffusion of the gas into the liquid, and the greater the rate of respiration one may measure without diffusion errors.

Dixon and Tunnicliffe (1923) and Dixon and Elliott (1930) have studied these effects in the Barcroft differential manometer (see Chapter 7) and have concluded that 600-700 μl. O_2 per hour can be safely measured without diffusion errors when a shaking rate of 100 oscillations per minute is employed (over 1500 μl.O_2/hr. at a rate of 138 oscillations per minute).

In the Warburg respirometer the flasks used are usually smaller so the surface exposed to the gas is less than in the Barcroft type; hence, limiting rates of oxygen uptake are reached sooner. The actual rates measureable without errors arising from gas diffusion in flasks of approximately 15 ml. volume containing 3 ml. of fluid have been determined in the experiment described below. This illustrates one method for determining whether the rate of oxygen diffusion is the limiting factor in any results one might obtain (Fig. 5)

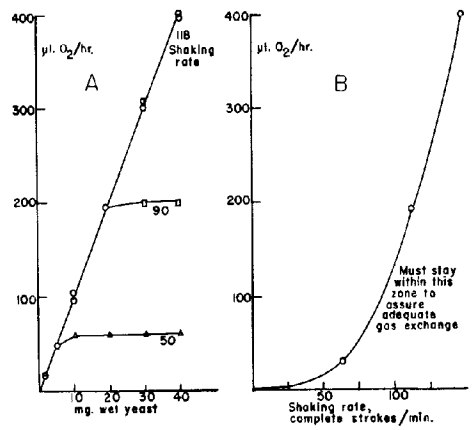

FIG. 5

Influence of shaking on rate of oxygen uptake in flasks of about 15 ml. capacity. Section A - Each flask has amount of yeast indicated on abscissa made to 2 ml. with 0.02 M KH_2PO_4 (pH 4.8), 1 ml. of 3% glucose, and 0.2 ml. 20% KOH in center well. Shaken at 50, 90 and 118 complete two-centimeter strokes per minute at 28°C. Section B - Data from Section A plotted to show adequate shaking rate for conditions described.

The basis of the experiment described is as follows: the rate of oxygen diffusion from the gas phase into the fluid phase is dependent upon the surface boundary. This boundary is altered more rapidly with faster shaking allowing more rapid oxygen exchange. If more rapid shaking (and thus more rapid oxygen exchange) does not increase the rate of oxygen uptake, then the rate of oxygen diffusion is not the limiting factor in the system being studied.

Another principle which may be used (but only in certain circumstances) depends upon the fact that the higher the concentration of the gas the greater will be its rate of diffusion into a liquid. Hence, one can vary the percentage oxygen in the atmosphere above the liquid, use various quantities of tissue, and determine the maximum rate of oxygen uptake that can be achieved before diffusion factors become significant. This method is, however, not only more laborious but also more complex, for there are reports that some

types of respiration are affected by the pressure of oxygen, per se. Therefore changing the shaking rate is the preferred method. Increasing the oxygen pressure is useful, however, when one finds it necessary to supply adequate concentrations of oxygen throughout a solid tissue. In this case the diffusion into the liquid is not the limiting factor, but diffusion into the respiring solid controls the oxygen level at its center. Obviously increased shaking will not alter these surfaces, so that the only practical solution is to increase the oxygen pressure. This is discussed under "tissue slices" in Chapter 10.

It sometimes happens that a reaction is dependent upon a contact between particles and that shaking disturbs this contact. One such example reported is the sulfur oxidation by bacteria (Vogler, LePage and Umbreit, 1942) in which a contact between the bacteria and the solid sulfur particles is necessary before oxidation can occur (Vogler and Umbreit, 1941, Umbreit, Vogel and Vogler, 1942). Shaking at rapid rates actually disturbs such contact and results in lowered oxidation. However, it is notable that if one employs rates of oxygen uptake lower than those at which limited diffusion becomes significant (i.e., at 100 strokes per minute, 300 μl.O_2/hr.) any variation in the rate of oxygen uptake with increase in shaking rate is not dependent upon the diffusion of oxygen since the fluid is already saturated. Therefore it is always desirable to determine the effect of alterations in the shaking rate to be certain that the results are independent of the rate of shaking. If they are not, one can readily determine whether the shaking rate is affecting the diffusion of oxygen or other factors (such as contact) by comparison with the rate of oxygen uptake which can be measured without diffusion effects under the conditions employed. Frequently important clues as to the nature of the reactions involved are obtained in this way.

THE ABSORPTION OF CARBON DIOXIDE

In the "direct method" of Warburg the oxygen uptake by living tissues, which also liberate CO_2, is measured by absorbing the CO_2 continuously in alkali during the determination. If the alkali employed fails to absorb the CO_2 completely and instantaneously, the CO_2 pressure in the gas phase will not be zero, and the readings on the manometer will not represent the true oxygen uptake. An example of circumstances of this type is given in the report of Brock, Druckrey, and Richter (1939); they observed that because of the large amounts of CO_2 liberated, readings on the manometer dropped only slightly or in some instances actually rose, yet oxygen was being consumed at a rapid rate. These workers found that the absorption of CO_2 was virtually instantaneous and that its pressure was held at approximately zero if the rate of CO_2 liberation was not more than 600 μl. per hour. Dixon and Elliott (1930) found that in the Barcroft apparatus (in the presence of adequate surface, see below) 1000 μl. of CO_2 per hour was almost instantaneously absorbed.

In absorbing CO_2 from the gas phase the same difficulties are encountered as in the absorption of oxygen. Here, however, because alkali is usually confined to the small center cup, an increased rate of shaking has little effect on increasing the surface. Hence some other method must be employed to increase the surface of the alkali. Usually small rolls or accordion folded pieces of filter paper are placed in the alkali cup. These should project beyond the side walls of the center cup into the open gas space above. A desirable projection is about 5 mm. Such "KOH papers" are usually prepared in quantity by cutting filter paper into squares with 2 cm. sides (the exact dimensions will vary with the depth of the cup employed; this varies from instrument to instrument, but the size need be only approximate). These papers are then folded three or four times, accordion fashion, and inserted into the center cup with tweezers. When wet by the alkali, previously added to the cup, they provide a large surface for the absorption of CO_2.

Sufficient alkali should be added to moisten the entire paper and still leave a well of free liquid in the bottom of the cup. For the papers described 0.15 to 0.20 ml. is adequate. Sometimes difficulty is experienced with the alkali "creeping over" the cup into the outer compartment of the flask. This can be prevented by greasing the top of the cup before inserting the papers. A convenient way of doing this is to wind a small amount of cotton about the end of a glass rod so that when placed over the center cup it will completely cover its top. After the cotton is saturated with grease, it is rotated in contact with the top surface of the center cup to give it a light coat of grease. A tapered 15 ml. centrifuge tube also makes a convenient tool for greasing alkali cups; the

bottom of the tube is greased and then rotated in the top of the alkali well. The relation of flask design to "creeping" of alkali is discussed in Chapter 6.

The concentration of alkali employed by various investigators differs widely, but KOH (because of the solubility of the potassium carbonate) is almost universally employed. Two things must be kept in mind in choosing the concentration. One is the capacity of the alkali employed to absorb CO_2; the other is the ease with which the alkali can be handled. Whereas the pressure of CO_2 above any solution of KOH is zero, very dilute solutions of KOH may be completely neutralized rather soon. Under most circumstances 1% KOH is undoubtedly sufficient. Most workers use 5, 10 or 20% KOH to be sure that an adequate supply is present to last throughout the experiment. 20% KOH offers no difficulty in handling. It is claimed by some that rather concentrated solutions of KOH (10-20%) react with the filter papers employed and that an oxygen uptake results from this reaction. While we have never experienced this, the recommendation that analytical grade filter papers be used for KOH papers should be followed whenever possible.

It is obvious that the conditions for obtaining adequate oxygen diffusion and CO_2 absorption are easily met. Usually the shaking rates employed are 100 to 120 two or three centimeter strokes per minute. Under these conditions (employing flasks of about 15 ml. capacity) one should use amounts of tissues that take up less than 300 μl. of O_2 per hour and give off less than 500 μl. CO_2. This usually means the use of about 100 mg. (wet weight) of animal tissues or somewhat less wet weight of yeast and bacteria. For the beginner it is well to choose tissue concentrations which take up about 200 μl. O_2 per hour.

PROCEDURE EMPLOYED

The actual procedure in setting up systems for the measurement of respiration of living cells varies widely. A common procedure is listed as follows:

1. To clean, dry, Warburg flasks equipped with a center well, add materials (except cells) to the main compartment of flask.
2. Add materials (if any) to the sidearm.
3. Add 0.2 ml. alkali (usually 5, 10 or 20% KOH) to the center well.
4. Grease attachment joint on manometer and grease and insert plug for sidearm. Grease top of alkali cup.
5. Add cells.
6. Add filter paper strip to alkali in center cup (see absorption of carbon dioxide).
7. Attach flask to manometer.
8. Place in constant temperature bath.
9. Adjust and tighten flask after about 5 min. shaking in bath. (This is done since sometimes the grease becomes softer and the flask tends to creep slightly.)
10. Allow to equilibrate, with shaking, for 10-15 minutes.
11. Adjust manometer fluid to zero point on closed side of manometer with stopcock open.
12. Close stopcock.
13. Begin readings.

LIMITATIONS OF METHOD

The method described in the previous sections, in which any carbon dioxide formed is absorbed by alkali, is known as Warburg's "Direct Method". It is the method most widely used for determining respiration. As with any other method it has certain limitations; these are:

1. The gases exchanged must be only O_2 and CO_2. In most cases this condition is not difficult to meet since in the majority of tissues these are the only gases involved. Warburg (1926), however, points out that "the metabolism of bacteria is rarely so simple that it can be measured by this method". This is a somewhat pessimistic viewpoint, and many bacteria can be studied adequately by this method. However, one should always take care to check that the only gases involved are O_2 and CO_2 before relying upon data derived by this method.

2. One must work in an atmosphere free from carbon dioxide. For some tissues, this is of no consequence, i.e. they respire at the same rate, to the same extent, and follow the same pathways whether CO_2 be present or not. But for others, this is by no means true. Carbon dioxide may inhibit, may stimulate, or may alter the path of metabolism of a given cell, hence measurements in the absence of CO_2 may not give a reasonable estimate of the reactions occurring in its presence. For this purpose the Warburg "Indirect Method" may be used (see Chapter 4).
3. The rate of oxygen uptake, and the rate of carbon dioxide liberation and absorption must be within a particular range so that the assumptions of the method hold, i.e., that the fluid is always saturated with oxygen gas (or air) and that the pressure of carbon dioxide in the gas phase is always zero.

Thus, in spite of the limitations of the "Direct Method" the conditions necessary for its adequate functioning can be met with ease in most cases.

RESULTS OF DETERMINATIONS

The Warburg "Direct Method" is suitable for two general types of use:

1. The determination of the rate of oxygen uptake.
2. The determination of the amount of oxygen uptake.

Both are usually measurable in the same determination. In expressing the rate of oxygen uptake, a quotient ("Q") is commonly employed. Several of these are in general use. These are defined as follows:

Q_{O_2} = μl. O_2 taken up per mg. dry weight of tissue per hour.

$Q_{O_2}(N)$ = μl.O_2 taken up per mg. tissue nitrogen per hour.

$Q_{O_2}(P)$ = μl.O_2 taken up per mg. tissue phosphorus per hour, or per mg. nucleic acid phosphorus per hour.

$Q_{O_2}(C)$ = μl.O_2 taken up per mg. tissue carbon per hour.

$Q_{O_2}(cell)$ = μl. O_2 taken up per cell per hour.

In short, one specifies in the Q term the conditions under which the rate was measured and the basis used to estimate the amount of tissue. In a general way:

$$Q \begin{matrix}(\text{gas atmosphere}) \\ (\text{gas measured})\end{matrix} (\text{tissue basis})$$

For example:

$Q^{O_2}_{O_2}(N)$ means μl.O_2 taken up per mg. nitrogen of tissue per hour in an atmosphere of pure oxygen.

$Q^{N_2}_{CO_2}(P)$ means μl.CO_2 given off in an atmosphere of nitrogen (or under anaerobic conditions) per mg. of tissue phosphorus per hour.

Uptake of a gas is indicated by a minus (-) sign, release by a plus (+). Two conventions are employed:

1. When the gas atmosphere is air, the condition indicator is omitted.
2. When the tissue basis is dry weight, this indicator is omitted.

Thus, the term Q_{O_2} is used rather than $Q^{air}_{O_2}(\text{dry weight})$, and $Q_{O_2}(N)$ means oxygen uptake in air per unit nitrogen per hour, while $Q^{O_2}_{O_2}(N)$ means oxygen uptake in pure oxygen per unit nitrogen per hour.

The various values which are employed (Q_{O_2} and $Q_{O_2}(N)$ being the most common) represent an effort to estimate the "active" portion of the cell constituents. For example, many bacteria produce a "gum", a carbohydrate material on the cell surface. This material is not "alive" (being usually a reserve carbohydrate that can be metabolized but slowly), yet it contributes to the cell volume, the wet weight, the dry weight, and, because it frequently occludes mineral matter from the medium, it even contributes to the ash. Because of this gum formation, the dry weight, etc. of such cells does not actually measure the active cell content. In such a case one then attempts to obtain a convenient measure of the amount of the active portion of the cell, usually nitrogen or phosphorus, or some other component which is not influenced by the mere accumulation of an "inert" storage product. The actual measure employed is dependent upon the tissue one is using, and it should be one closely connected with cell activity. For a further discussion see Burris and Wilson (1940) and Berenblum, Chain and Heatley (1939). Care must be taken, in comparing respiration rates (Q_{O_2} values) from tissues of different sorts, that inert materials have not contributed to the basis used and thus influenced the rate obtained.

It is also possible to compare the effect of the treatments without knowing the exact amount of tissue involved, as long as it is the same in all cases. Thus, one might use 1 ml. of a bacterial suspension (whose dry weight, nitrogen, etc. were not known) in each of a series of buffers at different pH's. The effect of pH could be observed without determining the exact amount of tissue.

The second use to which the "Direct Method" may be put is the determination of the amount of oxygen taken up per unit of substrate added, i.e., how many moles of oxygen are used in oxidizing x moles of substrate. For this purpose one usually employs flasks with sidearms. A known quantity of the material to be studied is placed in the sidearm. After equilibration, the rate of oxygen uptake is determined in the absence of substrate (to be certain that it is constant), the substrate is then tipped in, and the oxygen uptake determined until it again reaches the endogenous (respiration in the absence of substrate) rate. An example of this type of data is given in an experiment shown in Fig. 6.

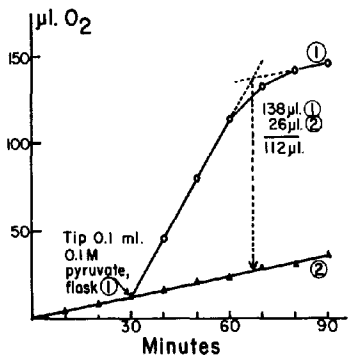

FIG. 6

Example of the use of the Warburg "Direct Method" to determine total oxygen uptake per unit of substrate added. Flasks contain: 0.5 ml. (containing 1.0 mg. cell nitrogen) E. coli suspension, 1 ml. M/10 phosphate buffer pH 7.0, 0.1 ml. 4% KOH in center well. Flask 1: 0.1 ml. 0.1 M (0.01 mM.) pyruvate neutralized to pH 7.0 in sidearm, 1.4 ml. water added to main compartment. Flask 2: 1.5 ml. water added to main compartment. O_2 taken up due to pyruvate addition = 138 - 26 = 112 µl. or 0.005 mM. O_2. As 0.01 mM. pyruvate was added, 0.5 O_2 was taken up per pyruvate molecule. This represents the removal of 2H from pyruvate or its oxidation to the acetate stage.

It will be noted that from this type of experiment it is possible to obtain both the amount of oxygen used per mole of substrate and the rate of oxygen uptake. For example, in the experiment cited, the rate of oxygen uptake can be calculated from the period of 30 to 60 minutes; $Q_{O_2}(N) = 204$. The rate obtained under these conditions may not be, however, the maximum rate possible, since in order to measure the oxygen taken up in a reasonable length of time one may find it necessary to add substrate in quantities insufficient to saturate the enzyme systems. Normally the enzyme systems are considered saturated if one obtains a straight line function with time, but occasionally instances may be found in which the rate of oxygen uptake (or other functions of metabolism) may proceed in a linear manner, yet higher levels of the substrate will increase the rate.

In determining the amount of oxygen taken up per unit of substrate, it is frequently a problem to decide whether one should subtract from the oxygen uptake observed in the presence of substrate, the oxygen taken up over the same interval in the absence of substrate. That is, when a substrate is being oxidized at a rapid rate, does the endogenous respiration continue at its constant rate, or is it suppressed, or does it increase? These questions have not yet been answered. Undoubtedly the response depends upon the tissue involved, and no generalizations can be made. However, it is always good practice to determine the endogenous respiration and to report it, along with the oxidation in the presence of substrate, and to indicate whether or not the endogenous respiration was subtracted from the substrate respiration in calculating the oxygen consumption per mole of substrate. See Van Niel (1943) for further discussion.

It is frequently convenient to express the amount of substrate employed in terms of gas produced or absorbed. Since 1 mole of any gas (at standard conditions) occupies 22.4 liters, it is possible to consider any substance in terms of liters with each mole equivalent to 22.4 liters. The following table (Table V) makes this clear. One may thus speak of adding 11.2 µl. of glucose, which means that one has added 0.5 ml. of 0.001 M glucose solution or 5×10^{-7} moles of glucose. This terminology may not be clear when a reaction releases a fraction or more than 1 mole of gas per mole of substrate. For example, the complete oxidation of glucose releases 6 moles of CO_2, and to speak of 0.5 ml. of 0.001 M glucose as 11.2 µl. of glucose when 67.2 µl. of CO_2 actually are released is confusing.

TABLE V

Relation between Concentration and Gas Volume

Unit	Contained in	0.1 M	0.01 M	Gas Volume in µl.
1 mole	1 liter of a 1 M soln.			2.24×10^7
1 millimole, 10^{-3} mole	1 ml. of a 1 M soln.	10 ml.	100 ml.	2.24×10^4
0.1 " , 10^{-4} mole	--	1 ml.	10 ml.	2,240
0.01 " , 10^{-5} mole	--	0.1 ml.	1 ml.	224
1 micromole, 10^{-6} mole	--	0.01 ml.	0.1 ml.	22.4

It is also possible to reverse this procedure and to determine µM of oxygen consumed rather than µl. One need only divide the µl. of O_2 consumed by 22.4 to obtain micromoles (10^{-3} millimoles) of the gas used. It is sometimes even convenient to employ a "Molar Flask Constant" rather than the usual flask constant such that the readings on the manometer may be directly converted into micromoles consumed. To obtain the "Molar Flask Constant" divide the usual flask constant by 22.4. It should be noted that what is measured on the manometer is O_2, not O, i.e., its molecular weight is 32.

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Chapter II

THE "DIRECT METHOD" FOR CARBON DIOXIDE

THEORY

In the previous chapter we noted that one could measure respiration even if carbon dioxide were given off, by absorbing all of the carbon dioxide in alkali. Therefore, if one had two flasks each respiring in exactly the same way, except that in one, the carbon dioxide was absorbed whereas in the other it was not, one would have a measure of the carbon dioxide liberated (Dixon, 1943; Warburg, 1926). There is one precaution which must be noted; one tissue (absence of alkali) is respiring in the presence of carbon dioxide, the other (with alkali) respires in its absence. If this should make a difference in the rate of oxygen uptake, or indeed in the course of the reactions followed, an error would be introduced. In most cases carbon dioxide has little effect upon the rate of respiration and methods will be described later (Chapter IV) which enable one to determine whether carbon dioxide is influencing the respiration rate and even to measure its rate under these conditions.

Using the symbols employed in Chapter I, the change in the manometer fluid level (h) read on the flask without alkali, is the resultant of decreasing pressure due to the absorption of oxygen and increasing pressure due to the liberation of carbon dioxide.

Manometer change due to oxygen absorption = $h_{O_2} = x_{O_2}/k_{O_2}$ since $x_{O_2} = h_{O_2}k_{O_2}$

Manometer change due to carbon dioxide production = $h_{CO_2} = x_{CO_2}/k_{CO_2}$

The final observed reading h in the flask without KOH, would be the resultant of the two, i.e.,

$$h = h_{O_2} + h_{CO_2} = x_{O_2}/k_{O_2} + x_{CO_2}/k_{CO_2}$$

hence

$$(1)\quad x_{CO_2} = (h - x_{O_2}/k_{O_2})\, k_{CO_2}$$

Now x_{O_2} is known from the flask which contained KOH, and k_{O_2} and k_{CO_2} are known for the flask without KOH, hence x_{CO_2} can be calculated.

Example: 1 ml. of an algal cell suspension was placed in each of two flasks together with 2 ml. of water. Flask 1 ($k_{O_2} = 0.96$) contained 0.2 ml. KOH, flask 2 ($k_{O_2} = 1.04$, $k_{CO_2} = 1.25$) had no KOH. After equilibrating, respiration was permitted for 30 minutes. In flask 1, the change in reading (h) was 28 mm., hence, 28 x 0.96 = 26.9 µl. O_2 taken up.

In flask 2, over the same interval, the manometer reading dropped 9.5 mm. hence,

$x_{CO_2} = (-9.5 - (-26.9)/1.04)\ 1.25 = (-9.5 + 25.8)\ 1.25 = 16.3 \times 1.25 = 20.4$ µl.CO_2.

The R. Q. (Respiratory Quotient = CO_2 produced /O_2 consumed) is in this case, 20.4/26.9 = 0.76.

In essence this method determines the oxygen uptake in the absence of CO_2 in one flask, and one then calculates what the change in reading should have been in the other flask if no CO_2 were produced. The uptake observed is always less than this amount, hence the difference is due to CO_2 liberation.

A convenient way of recording data and making the calculations is to use a chart of approximately the following construction (Table VI).

TABLE VI

Method of Calculating Results

Time	Flask with KOH		Flask without KOH				
	h	µl. O_2	h	$\frac{x_{O_2}}{k_{O_2}}$	diff.	µl. CO_2	R. Q.
	mm. observed change corrected for thermobarometer	corrected h times k_{O_2} for flask	mm. observed change corrected for thermobarometer	column 3 divided by k_{O_2} of this flask	substract column 5 from column 4	multiply column 6 by k_{CO_2}	divide column 7 by column 3
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
30 (from example cited)	-28	-26.9 k_{O_2}=0.96	-9.5	-25.8 k_{O_2}=1.04	+16.3	+20.4 k_{CO_2}=1.25	0.76

One may also substitute in equation (1) as follows. Equation (1) is:

(1) $x_{CO_2} = (h - x_{O_2}/k_{O_2})k_{CO_2}$, the constants referring to the flask without KOH

$x_{O_2} = h'k'_{O_2}$ where h' and k'_{O_2} refer to the flask with KOH, hence

(2) $x_{CO_2} = (h - h'k'_{O_2}/k_{O_2})k_{CO_2}$

Therefore one may calculate the amount of CO_2 released or absorbed during a given interval by subtracting from the change in reading on the flask with no alkali, the change in reading on the flask with the alkali times the ratio of the two oxygen constants, the whole times the CO_2 constant.

CORRECTIONS FOR UNEQUAL AMOUNTS OF TISSUE

It sometimes happens, in using plant or animal tissues, that one does not add exactly the same amount of tissue to each of the flasks. If the differences in amount of tissue are not large, one may merely divide the readings (h) of each flask by the weight (or other measure of active tissue), to obtain the uptake per unit of tissue. The readings obtained with the two flasks are thus comparable. Suppose that in the example listed, the flask with KOH contained 0.97 mg. algae whereas the flask without KOH contained 1.15 mg. algae. In flask 1, the change was 28 mm., or the change per mg. of tissue was $\frac{28}{0.97} = 28.9$; O_2 uptake was thus 28.9 x 0.96 = 27.7 µl.O_2 taken up per mg. tissue. In the flask without KOH the manometer reading dropped 9.5 mm.; 9.5/1.15 = 8.25 mm. per mg. of tissue. The two changes are now comparable since both are based on the same quantity of tissue, hence

$$x_{CO_2} = (-8.25 - (-27.7)/1.04)\ 1.25 = (-8.25 + 26.6)\ 1.25 = 18.35 \times 1.25$$

$$= 22.9\ \mu l.CO_2 \text{ produced; R. Q.} = 22.9/27.7 = 0.83$$

CORRECTIONS FOR RETENTION IN BUFFERS

When buffers are present they react with CO_2, for example:

$$Na_2HPO_4 + CO_2 + H_2O \rightleftharpoons NaH_2PO_4 + NaHCO_3$$

Hence the CO_2 which may escape to the air (and be measured as x_{CO_2}) may be less than that actually produced in the intervals measured. As will be shown in Chapter 3, at a pH of 5 or below no appreciable amount of HCO_3^- exists, hence if the solution is adjusted at the end of the reaction to a pH of 5 or below, all such "bound CO_2" will be released. Therefore in order to obtain the total CO_2 liberated in the presence of buffers, one tips in acid from the sidearm and releases the CO_2 from the buffer. Since the tissue or the buffer may have contained bound carbon dioxide initially, three manometers are used as follows:

(1) + KOH to determine x_{O_2} (h)

(2) - KOH + acid added at end (h_2)

(3) - KOH + acid added at start (h_3)

The h_3 represents the initial bound CO_2, while h_2 represents that initially bound + that released during the respiration. One can obtain the actual CO_2 evolved as follows:

Initial bound $CO_2 = h_3 k_{3CO_2}$

Initial bound CO_2 + CO_2 evolved = $h_2 k_{2CO_2}$

Hence, CO_2 evolved is $h_2 k_{2CO_2} - h_3 k_{3CO_2}$

Thus, if one takes the readings after the acid is added, the h of equation (1) is not h_2 but $h_2 - h_3 \frac{k_{3CO_2}}{k_{2CO_2}}$. Hence the equation (1) becomes $x_{CO_2} = \left[h_2 - h_3 \frac{k_{3CO_2}}{k_{2CO_2}} - \frac{x_{O_2}}{k_{2O_2}}\right] k_{2CO_2}$

A very useful method of correcting for CO_2 retention has been suggested by M. J. Johnson. Since at high pH values HCO_3^- is held in solution as well as CO_2, the effective value of α (which will be designated as α') will be larger than the true α value. Since pK'_a from the apparent first dissociation constant for carbonic acid is 6.317 at 38°C. (Shedlovsky and MacInnes, 1935),

$$\frac{\alpha'}{\alpha} = \frac{(HCO_3^-) + (CO_2)}{(CO_2)} = \left[\text{antilog } (pH - 6.317)\right] + 1$$

where the pH is that in the reaction flask during the experiment. If the flask constant used is calculated by the use of α' instead of α, retention of CO_2 will be corrected for automatically. As α' increases rapidly with pH, the retention correction becomes very large at pH values above 7, and the accuracy of the CO_2 measurement suffers accordingly. Values for α'/α at 30° and 37° C. may be obtained from Fig. 7.

RESPIRATORY QUOTIENTS

These quotients are defined as the ratio CO_2 produced/oxygen consumed, and they serve to indicate the nature of the metabolism. While an R. Q. of 1 would occur upon complete oxidation of carbohydrate (0.9 for most proteins, 0.8 for most fats) the finding of these values does not thereby prove that metabolism of carbohydrate, protein, etc., is the cause of the R. Q. Nevertheless, the R. Q. is an index of the processes occurring and should be measured if possible. Later sections (especially Chapter 8) will describe other methods of determining this value.

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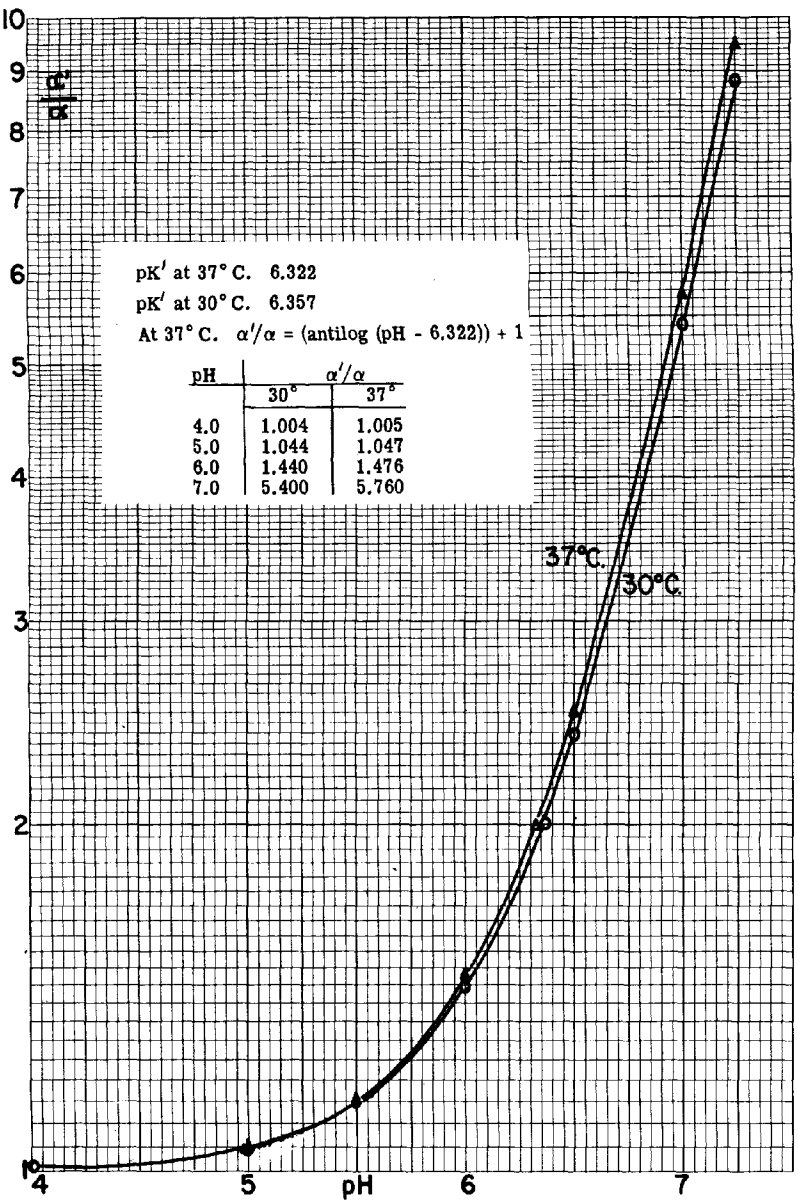

FIG. 7

Ratios of apparent to real solubility of carbon dioxide.

Chapter III

CARBON DIOXIDE AND BICARBONATE

THE SOLUBILITY OF CARBON DIOXIDE

The solubility of carbon dioxide in pure water is in essence no different from the solubility of other gases. While it is true that the carbon dioxide forms carbonic acid which dissociates to form H^+ and HCO_3^- in accordance with equation (3),

$$(3)\quad CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

it is also true that over 99% of carbon dioxide in solution is in the form of dissolved carbon dioxide and less than 1% exists as H_2CO_3, H^+ or HCO_3^-. In the absence of materials which can combine with the acid, the solubility is comparable to that of any other gas. There are two factors which render the actual figures obtained on solubility in pure water somewhat more variable than those for other gases. One is the somewhat higher Van der Waals forces which exist in carbon dioxide, which is equivalent to saying that carbon dioxide deviates from the laws of an ideal gas somewhat more than other gases, but the error involved in manometric work is negligible. The other factor is that in dissolving in pure water, H^+ ions are generated so that the pH does not remain at 7 but gradually decreases as the pressure of carbon dioxide is increased. The α values for carbon dioxide are given in Table VII.

TABLE VII

The Solubility of Carbon Dioxide in Pure Water

Data in terms of α = ml.CO_2/ml. water or μl.CO_2/μl. water at one atmosphere

Temp. °C.	(1)	(2)	(3)
0	1.713		
10	1.194	1.194	
15	1.019	1.019	1.014
20	0.878	0.878	
25	0.759		0.756
30	0.665	0.66	
35	0.592		
40	0.530	0.53	

(1) Handbook of Chemistry and Physics (1944)
(2) Dixon (1943)
(3) International Critical Tables (1928)

THE INFLUENCE OF SALTS ON CO_2 SOLUBILITY

As was shown in the case of oxygen, the presence of other salts, etc., in solution has little effect upon the solubility of carbon dioxide, within physiological concentrations, providing that these do not combine with the carbonic acid. As is shown in Table VIII the effect of various salts, although greater than with oxygen, is negligible.

THE INFLUENCE OF CARBONATE AND BICARBONATE

If, however, there is anything in the solution which will combine with the carbonic acid (or bicarbonate ion), the entire solubility changes. Since from equation (3):

(3) CO_2 (gas) $\rightleftharpoons$ CO_2 (dissolved) $\rightleftharpoons$ H_2CO_3 $\rightleftharpoons$ $H^+ + HCO_3^-$

and since H_2CO_3 is essentially dependent upon CO_2 (dissolved) which is, in turn, directly dependent upon the pressure of carbon dioxide (pCO_2) in the gas phase, the equilibrium

TABLE VIII

The Influence of Salts and Other Materials upon the Solubility of Carbon Dioxide

Material	At 15°C (α values)			At 25°C (α values)		
	0.5 M	1.0 M	2.0 M	0.5 M	1.0 M	2.0 M
None	1.014	--	--	0.756	--	--
HCl	0.989	0.974	0.948	0.738	0.732	0.728
1/2 H_2SO_4	0.965	0.927	0.867	0.727	0.705	0.669
HNO_3	1.022	1.029	1.043	0.770	0.781	0.803
KCl	0.925	0.850	--	0.695	0.641	--
NH_4Cl	--	--	--	0.720	0.692	0.648
Glycerol	--	0.934	--	--	--	--

From International Critical Tables (1928)

constant of the dissociation of the carbonic acid, which would normally be written as equation (4a), becomes, in reality equation (4):

(4a) $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$ $\qquad K_1' = \dfrac{(H^+)(HCO_3^-)}{(H_2CO_3)}$

K_1' indicates the first dissociation constant of carbonic acid.

(4) $\qquad K_1 = \dfrac{(H^+)(HCO_3^-)}{(CO_2)}$

One may solve this equation (4) for H^+ as follows:

$$(H^+) = \frac{K_1(CO_2)}{(HCO_3^-)}$$

If one takes the logarithms of both sides:

$$\log (H^+) = \log \frac{K_1(CO_2)}{(HCO_3^-)}$$

But a $-\log (H^+)$ is termed pH, so

$$pH = - \log \frac{K_1(CO_2)}{(HCO_3^-)}$$

But also, since $\log xy = \log x + \log y$ (from definition of logarithms)

$$pH = - \log K_1 - \log \frac{(CO_2)}{(HCO_3^-)}$$

In addition, since $\log \frac{x}{y} = - \log \frac{y}{x}$, the equation immediately above becomes:

$$(5)\quad pH = - \log K_1 + \log \frac{(HCO_3^-)}{(CO_2)} = - \log K_1 + \log \frac{\text{bicarbonate}}{\text{carbon dioxide}}$$

One may note the similarity of the term pH to express - log (H^+), to the -log K_1 occurring in equation (5). Hence the expression pK_1 is quite logical and a convenient way to express the value - log K_1. We can therefore define the term:

$$pK_1 = - \log K_1$$

As long as such substitutions are being made, we can include in the pK_1 values employed, another factor to account for the "activity" of the materials involved which is not exactly the same as their concentration. We can thus define a pK' as follows:

$pK' = pK_1 + \log \theta$ where θ is the activity coefficient of the HCO_3^-.

This will make the equation just a little more exact. Employing this value, then, equation (5) becomes:

$$(6)\quad pH = pK' + \log \frac{(HCO_3^-)}{(CO_2)}$$

This equation (6) is sometimes called the "Henderson-Hasselbach equation" relating the CO_2 pressure, the bicarbonate ion concentration and the pH. Naturally, pK' must be known. This value is given by Hastings and Sendroy (1925) at infinite dilution (pK'_∞) as 6.33 at 38°C., by MacInnes and Belcher (1933) as 6.343 at 25°C., 6.309 at 38°C., and by Shedlovsky and MacInnes (1935) as 6.317 at 38°C. The evidence cited by Shedlovsky and MacInnes (1935) points to 6.317 at 38°C. as the most probable value, and pK'_∞ will be taken as 6.317 at 38°C. in the subsequent discussion.

In concentrations of bicarbonate greater than infinite dilution the value of pK' will decrease slightly, the decrease being theoretically $6.317 - \sqrt{0.5\ \mu}$. The determinations of MacInnes and Belcher (1933) show this decrease to be 0.08 μ (where μ = the ionic strength). In the case of manometric measurements the highest bicarbonate concentrations employed are of the order of 0.1 molar. Since this alters the pK' by less than 0.008, the correction is so small that it can be ignored for most measurements.

At temperatures lower than 38°C. the pK' will increase. The increase due to temperature is given as 0.005 units per degree centigrade by Stadie and Hawes (1928). Shedlovsky and MacInnes (1935) found that the increase was not strictly linear, but from their data the following corrections may be applied: between 20 and 40°C. the increase due to temperature is 0.005 units / °C.; between 10 and 20°C., 0.006; between 0 and 10°C., 0.010. For virtually all manometric work the value 6.317 (38°C.) is sufficiently accurate, and this value will increase by 0.005 units per °C. as the temperature is lowered.

This equation (6) has very wide use, but it is subject to certain limitations. First, it neglects the second dissociation constant of carbonic acid (corresponding to the reaction $HCO_3^- = H^+ + CO_3^=$). However, E. J. Warburg (1922) has shown that if the pH is less than 8, the error arising from this neglect is unimportant. Second, the equation itself employs a term for bicarbonate concentration, (HCO_3^-), whose concentration one may not be able to determine exactly. For example, if $NaHCO_3$ is present, there will be HCO_3^- ion from the sodium bicarbonate and also bicarbonate ion from the dissociated carbonic acid (i.e., dissolved and dissociated CO_2). But again, E. J. Warburg (1922) showed that if the H^+ concentration was one one-hundredth (1/100) of the concentration of $NaHCO_3$ (or other metal bicarbonate), the bicarbonate concentration for use in the equation (6) could be taken as equivalent to that of the bicarbonate added. Neglect of the bicarbonate from carbonic acid, under these conditions, would cause an error of less than 1 part in 1000. Thus at pH 5 ($H^+ = 10^{-5}$M), the lowest bicarbonate concentration which could be employed would be 10^{-3}M (M/1000). At pH 7, the lowest bicarbonate concentration which could be

employed would be 10^{-5} M (M/100,000). It is obvious that in the physiological range of pH 6-8 it would not be difficult to supply sufficient bicarbonate to overcome any error arising from the neglect of the second dissociation constant of carbonic acid.

In actual practice, using contemporary Warburg instruments (overall accuracy of 5%) it is found that if bicarbonate salts are employed, one can use the concentration of the bicarbonate supplied as the (HCO_3^-) in the equation, providing the bicarbonate concentration supplied is at least ten times that of the H^+ concentration. (pH 6, $H^+ = 10^{-6}$ M, bicarbonate must be at least 10^{-5} M; at pH 8, $H^+ = 10^{-8}$ M, bicarbonate must be at least 10^{-7} M). In addition, under most practical circumstances one may use the bicarbonate supplied as the total bicarbonate concentration providing it is at least ten times the concentration of other "carbon dioxide binding" materials.

The carbon dioxide concentration in equation (6) is expressed in the same units as the bicarbonate, i.e., in moles per liter. Since the value usually known is the percent carbon dioxide, the following equation (7) is used to convert percent of carbon dioxide in the gas into moles per liter in the solution.

$$(7)\quad \text{carbon dioxide in moles per liter of solution} = \frac{P\,\alpha\,CO_2 \cdot 1000}{760 \cdot 22{,}400 \cdot 100}$$

where P = atmospheric pressure
CO_2 = percent of CO_2 at the atmospheric pressure, P
α = solubility of CO_2

Or simplifying:

$$(7)\quad \text{CO_2 in moles per liter of solution} = P\,\alpha\,CO_2 \cdot 0.587 \cdot 10^{-6}$$

The term P/760 converts the atmospheric pressure to standard conditions, α represents the solubility in the solution involved, 1000/22,400 is a factor to change α from liters/liter to moles/liter, and 100 in the denominator converts percent CO_2 to pCO_2.

Occasionally one will find equation (6) and (7) combined (as in equation 8).

$$(8)\quad pH = pK' + \log HCO_3^- - \log P\,(CO_2) - \log \frac{\alpha}{760 \times 2240}$$

If carbon dioxide is expressed in terms of mm. Hg pressure (mm. CO_2) the equation may be written as equation (9):

$$(9)\quad pH = pK' + \log HCO_3^- - \log \text{mm. } CO_2 - \log \frac{\alpha}{760 \times 22.4}$$

The "Henderson-Hasselbach equation" shows that in order to measure CO_2 at pH 7 there must be CO_2 in the atmosphere, since if the CO_2 pressure is 0, or approaches 0, the factor $\log (HCO_3^-)/(CO_2)$ (equation (6)) becomes larger (hence the pH increases) or, if the pH is held low, the HCO_3^- becomes zero. At a pH of 5 or below, no appreciable amount of bicarbonate or carbonate ion can exist, hence any CO_2 released will escape to the air. Thus one can measure CO_2 evolution from urea under the action of urease (which can occur at pH 5) without supplying CO_2 to the atmosphere. But most physiological reactions occur at pH 7, hence because of low levels of CO_2 in the air, either the bicarbonate concentration must be kept low, or CO_2 must be supplied in the air (if the pH is to be maintained at 7). It is obviously impossible to keep the bicarbonate low because the reaction of carbonic acid with tissue buffers tends to increase bicarbonate. The error arising from this source is only negligible in practice when the bicarbonate concentration is at least 10 times higher than that of tissue buffers. Hence the practical solution is to add CO_2 to the gas phase. It should be emphasized that if one fixes the bicarbonate concentration, one can obtain any pH between the values of 6 and 8 by adjusting the pCO_2 and vice

versa. We shall work out several examples, but it is obvious that the situation can be altered to fit many other cases.

PRACTICAL USE OF THE HENDERSON-HASSELBACH EQUATION

As described in the paragraph above, it is necessary to add CO_2 to the atmosphere under most operating conditions. It is convenient to fix the CO_2 level at 5% and to vary the bicarbonate concentration to obtain the pH desired. This concentration of CO_2 requires bicarbonate concentrations between 0.001 and 0.10 molar, and under these circumstances the amount of carbonate in solution is negligible. It is apparent that equation (6)

$$(6) \quad pH = pK' + \log \frac{(HCO_3^-)}{(CO_2)}$$

may be solved for $\log (HCO_3^-)$ and pH as follows:

$$pH = pK' + \log (HCO_3^-) - \log (CO_2) \qquad (\text{as } \log \frac{x}{y} = \log x - \log y)$$

$$- \log (HCO_3^-) = -pH + pK' - \log (CO_2)$$

$$(10) \quad \log (HCO_3^-) = pH - pK' + \log (CO_2)$$

In equation (10) $\log (CO_2)$ is expressed in moles/liter, thus substituting equation (7) into (10) one obtains (11):

$$(11) \quad \log (HCO_3^-) = pH - pK' + \log \frac{P \alpha\ CO_2}{760 \times 2,240}$$

If one fixes the CO_2 at 5% the last term becomes:

$$\log \frac{P \alpha\ 5.0}{760 \times 2,240} = \log P \alpha\ (2.94 \times 10^{-6})$$

At atmospheric pressure the P of course is dependent upon the exact pressure in the room; this pressure varies from day to day. However, the contribution of P to establishing the final concentration of bicarbonate is relatively small. For example, at 37°C. and pH 7.0 a change of P from 760 to 740 mm. Hg changes the bicarbonate required to obtain pH 7.0 from 0.00595 M (760 mm.) to 0.00588 (740 mm.). As the temperature is lowered this difference becomes slightly larger, e.g., at 20°C. 0.00786 M bicarbonate is required at 760 mm. and 0.00762 M at 740 mm. However, the change is still relatively small. In the following sections the data have been calculated for 740 mm. Hg pressure. Day to day alterations have relatively slight effect and only for the most precise work are any further corrections necessary. In the ordinary operation of the Warburg instrument the error introduced by working at pressures other than 740 mm. is so small that it can be ignored.

One may note that by specifying the conditions (5% CO_2, pressure between 760 and 720 mm. Hg calculated to 740 mm. Hg) equation (11) becomes:

$$\log (HCO_3^-) = pH - pK' + \log \alpha\ (2.17 \times 10^{-3})$$

The following table (Table IX) gives the bicarbonate concentration required at various temperatures to obtain the pH listed. The following two things may be noted:

First, at pH 7 the concentration of bicarbonate is some value (approximately 6) x 10^{-3} molar. At pH 6 it is this same value x 10^{-4} molar. At pH 8 it is the same value x 10^{-2} molar. Thus one need know only the concentration for one pH to know also the concentration required for 1 unit lower or 1 unit higher.

Second, the relationship between temperature and bicarbonate concentration is a linear one and at pH 7 for each degree below 37°C. the concentration of bicarbonate increases by 1 x 10^{-4} molar.

TABLE IX

Relation between Bicarbonate Concentration and pH.
(5% CO_2, atmospheric pressure of 740 mm. Hg.)

Temp. °C.	α CO_2	pK'	Molar Bicarbonate Concentration		
			pH 6	pH 7	pH 8
20	0.878	6.392	7.6×10^{-4}	7.6×10^{-3}	7.6×10^{-2}
25	0.759	6.365	7.1	7.1	7.1
30	0.665	6.348	6.6	6.6	6.6
35	0.592	6.328	6.1	6.1	6.1
37	0.567	6.322	5.88	5.88	5.88
40	0.530	6.312	5.68	5.68	5.68

These facts make possible the construction of a chart (Fig. 8) which facilitates the rapid determination of the bicarbonate concentration required. The line for 37°C. has been drawn in, since this is the temperature usually employed; however, concentrations required at any other temperature (between 20 and 40°C.) may be read by connecting the temperature scales at either end of the chart with a straightedge. A few examples given below will illustrate its use; with the chart the practical selection of the bicarbonate concentration for the pH desired becomes very simple. The chart itself is sufficiently accurate to furnish the degree of precision required for studies with the respirometer.

Examples:

(1) At 37°C. what bicarbonate concentration is necessary to obtain a pH of 7.2, 7.6, 6.8?

7.2 9.2×10^{-3} M = 0.0092 M

7.6 23×10^{-3} M = 0.023 M

6.8 36.7×10^{-4} M = 0.0036 M

(2) At 25°C. what bicarbonate concentration is necessary to obtain a pH of 7.2, 6.8?

7.2 11×10^{-3} M = 0.011 M

6.8 45×10^{-4} M = 0.0045 M

(3) At 37°C. and in 3 ml. liquid a reaction starting at pH 7.4 (14.5×10^{-3} M bicarbonate) evolves, from acid production, 200 µl. of CO_2 and then stops abruptly even though considerable substrate remains. At what pH did the reaction stop?

200 µl. of CO_2 represents 200/22.4 or approximately 9 µM CO_2 = 9×10^{-6} moles. This amount was produced in 3 ml. of liquid. Each ml. of the bicarbonate solution at the start had $\frac{14.5 \times 10^{-3}}{10^3} = 14.5 \times 10^{-6}$ moles of bicarbonate. As 9×10^{-6} moles total or 3×10^{-6} moles/ml. have been used, the amount remaining is $(14.5 - 3) \times 10^{-6}$ or 11.5×10^{-6} moles/ml. This is equivalent to 11.5×10^{-3} moles/liter and corresponds to a 11.5×10^{-3}-molar concentration. By referring to the chart it is apparent that the pH corresponding to this concentration is 7.3. In general terms:

$$\begin{matrix}\text{original molar} \\ \text{concentration} \\ \text{of bicarbonate}\end{matrix} - \frac{\mu l. \times 10^{-3}}{\text{fluid volume in ml.} \times 22.4} = \begin{matrix}\text{final molar} \\ \text{concentration} \\ \text{of bicarbonate}\end{matrix}$$

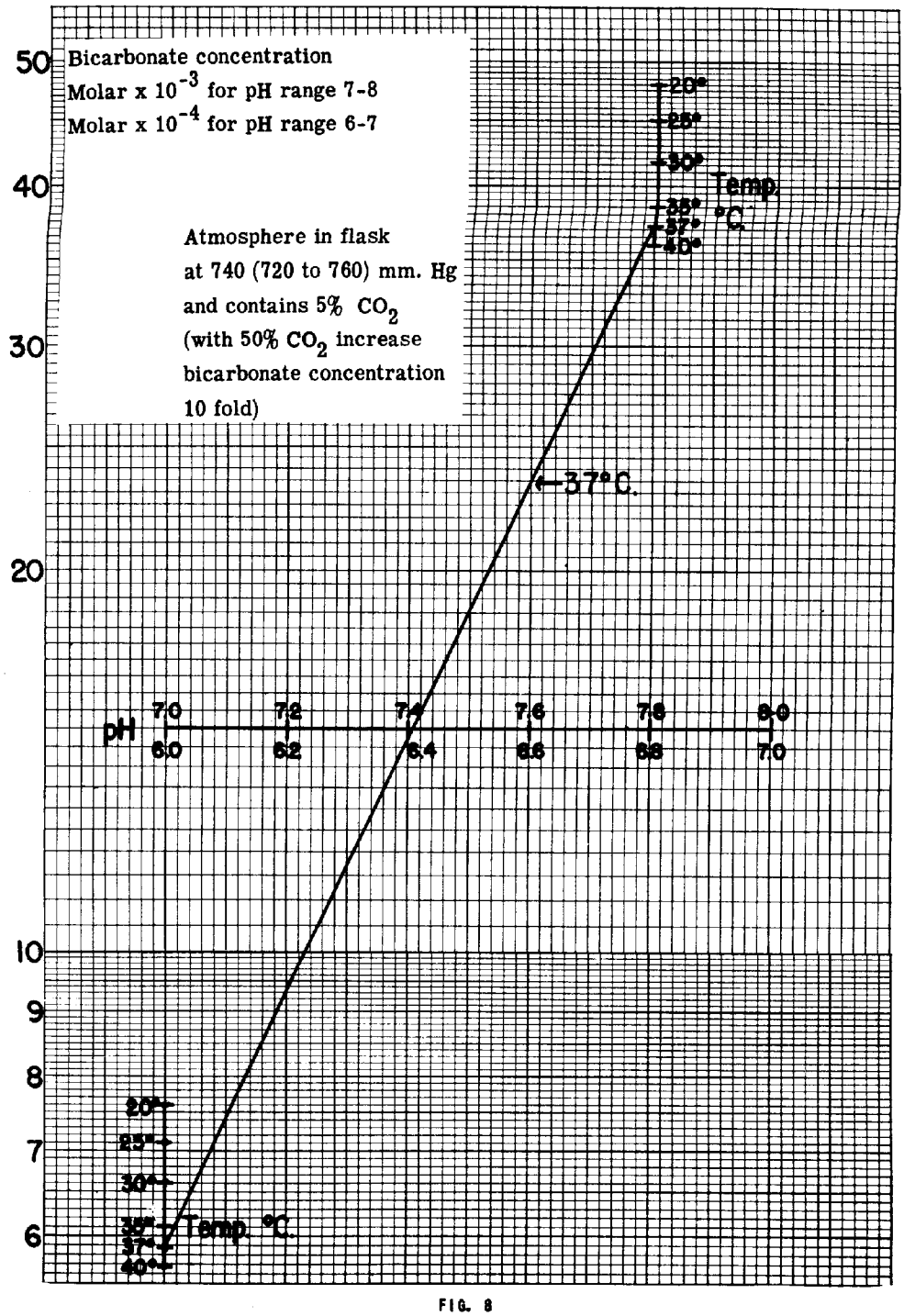

FIG. 8

Chart for determining the proper bicarbonate concentration to use at a given pH, pCO_2 and temperature.

THE USE OF BICARBONATE BUFFERS FOR MEASURING ACID PRODUCTION UNDER ANAEROBIC CONDITIONS

In the absence of oxygen there can be no respiration (i.e., oxygen uptake). With a bicarbonate buffer and an atmosphere containing CO_2, as described in the previous section, CO_2 released by the cells will escape into the atmosphere and can be measured manometrically. If any acid is produced under these conditions it combines with metallic ions formerly associated with bicarbonate, and as a result CO_2 is released. Hence, in bicarbonate buffers and under anaerobic conditions both metabolic CO_2 and CO_2 liberated through production of acid can be measured. Procedures for obtaining anaerobic conditions are described in Chapter 5.

If one employs a tissue which does not liberate CO_2 when metabolizing anaerobically, it is simple to determine the acid produced since one can take the CO_2 released from bicarbonate as a measure of the acid production. Another way which has been used to differentiate the "fermentation" CO_2 from CO_2 released by acid, is to determine the CO_2 production in the absence of bicarbonate and to subtract this from the CO_2 produced in its presence. However, the determination of the rate of CO_2 production in the absence of bicarbonate is unsatisfactory with most tissues, since to avoid any great effect of bicarbonate produced by interaction with buffers ("CO_2 retention") one must work in low concentrations of buffers. The CO_2 and acid liberated soon reduce the pH from the initial point and frequently stop metabolism.

A method which permits one to differentiate between the "metabolic CO_2" and CO_2 released by acid (both being measured as CO_2 liberated under anaerobic conditions) is the following:

Liberation of CO_2 will have little influence on the bicarbonate concentration, as the amount liberated is small relative to that in the gas phase and will not alter the pCO_2 appreciably. However, any acid which is liberated will produce CO_2 from bicarbonate, and the amount of bicarbonate will decrease. By adding sufficient acid to measure the bicarbonate remaining one can estimate the acid and the metabolic CO_2. Two manometers (plus a thermobarometer) are required. Each flask has tissue, buffer, bicarbonate, and a known pCO_2, and the gaseous and liquid phases are in equilibrium. Each has acid (usually 0.1 - 0.5 ml. 3N H_2SO_4) in the sidearm or in "Keilin tubes" (see Chapter 5). This quantity of acid is sufficient to stop the metabolism instantly and to reduce the pH below 5 (usually between pH 1 and 2) upon addition. In some rare cases the tissues are resistant to acid, so a poison is added along with the acid. After equilibration the acid is added in one of the flasks. The increase in manometer reading times k_{CO_2} gives the total initial bicarbonate available measured as CO_2. CO_2 output is measured for the experimental period in the other flask. Acid is then tipped in. In this case the amount of CO_2 released by the acid is a measure of the residual bicarbonate. The difference between this and the initial bicarbonate gives the amount of CO_2 produced by acid formation. Any other CO_2 released is that produced by the tissue as CO_2 and does not represent acid production (Warburg, 1914, 1926).

The usual Warburg instrument with 15 ml. flasks is capable of measuring over 300 µl. of CO_2. Since 1 ml. of 0.001 molar $NaHCO_3$ will release 22.4 µl. of CO_2, one may use as much as 1 ml. of 0.01 M $NaHCO_3$ (which in 3 ml. total volume at 37°C. and with 5% CO_2 gives a pH of 6.75) and release all the CO_2 as gas by tipping in acid. This will not extend the fluid in the manometer beyond its graduated range, providing one starts the experiment with the open end of the manometer at a low level (below 100 mm.).

Acid production under anaerobic conditions is usually spoken of as "glycolysis", and when animal tissues are employed the acid is largely lactic acid. In other cases, however, it is not valid to make the assumption that the product is lactic acid or that it is largely lactic acid. Hence, calculation of the acid produced as lactic is likely to result in error. Fortunately there is now available a very specific chemical method for the determination of lactic acid in the flasks (see Chapter 13), so that one can actually determine how much of the acid produced is lactic acid.

THE INFLUENCE OF CARBONATE

It has been pointed out that the "Henderson-Hasselbach equation" neglects the second dissociation constant of carbonic acid. In the presence of bicarbonate and CO_2, most of the Na^+ and K^+ ions are associated with bicarbonate but some carbonate does always exist. The question is, is there enough of this to make any difference? The CO_2 or acid produced will combine with carbonate and convert it to bicarbonate. This reaction releases no gas, so the CO_2 or acid involved will escape manometric estimation. The equations involved are:

$$(H^+)\ (HCO_3^-)/H_2CO_3 = (H^+)\ (HCO_3^-)/(CO_2) = K_1 = 3 \times 10^{-7}.$$

$$(H^+)\ (CO_3^{--})/HCO_3^- = K_2 = 6 \times 10^{-11}$$

$$K_1/K_2 = K' = \frac{(H^+)(HCO_3^-)}{CO_2} \Big/ \frac{(H^+)\ (CO_3^{=})}{HCO_3^-} = (HCO_3^-)^2/(CO_2)\ (CO_3^{--})$$

Thus $K' = K_1/K_2 = 3 \times 10^{-7}/6 \times 10^{-11} = 0.5 \times 10^{+4} = 5 \times 10^{+3} = 5000$

Using 5% CO_2 with a bicarbonate concentration of 0.01 molar at 25°C., the concentration of carbonate is:

$$CO_3^{--} = (HCO_3^-)^2/CO_2K' = (0.01)^2/1.52 \times 10^{-3} \times 5 \times 10^{-3} = 10^{-4}/5 \times 1.52 =$$

$$1.32 \times 10^{-5} \text{ moles } CO_3^{--}/\text{liter}$$

In short, this is such a small quantity that it may be neglected.

W. W. Umbreit

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Chapter IV

THE "INDIRECT METHOD" OF WARBURG

The one basic difficulty with the "direct" manometric methods described in Chapters 1 and 2, was that, in order to measure oxygen uptake, one was forced to work in an atmosphere free from CO_2. Yet for many tissues there was always the possibility that perhaps such measurements were not valid for this very reason, i.e., the rate of respiration, and even the course of the reactions, might be altered in the absence of CO_2. For example, decarboxylation reactions, which in the body tissue might be in equilibrium with CO_2, would be forced to completion if the CO_2 were all removed, and the influence of this situation upon the rate or course of oxygen uptake could not be estimated. Using the direct method there was no way of determining whether or not the presence or absence of CO_2 had an effect.

This problem was solved, at least partially, by Warburg (1924, 1926) who described a method which has since been termed Warburg's "Indirect Method". The principle upon which the method is based is that if one has two gases of markedly different solubility, one can measure the exchange of each by comparing the manometric changes when exactly the same reaction is carried out in two flasks which have either markedly different fluid volumes, or markedly different gas volumes.

PRINCIPLES AND DERIVATIONS

We shall confine ourselves to the discussion of a case in which one has two flasks of approximately equal volume containing markedly different volumes of fluid. It will be apparent that exactly the same equations hold for other circumstances (see later). We shall also confine the discussion to the gases O_2 and CO_2 (whose solubilities are markedly different), but it will be apparent also that any other gases may be substituted providing their solubility in the fluid in the flasks is different.

Suppose that we have two flasks of approximately the same volume, but containing different volumes of fluid. The same amount of tissue is placed in each and the same reaction occurs in each. In order to distinguish the two flasks, we shall use small letter symbols for the flask containing less fluid. (h = change in reading on manometer, k_{O_2} = flask constant for O_2, k_{CO_2} flask constant for CO_2, $-x_{O_2}$ = amount of O_2 taken up, $+x_{CO_2}$ = amount of CO_2 liberated, etc.) and capital letters (H, K_{O_2}, K_{CO_2}, X_{O_2}, X_{CO_2} etc.) for the flask containing the larger amount of fluid. This is purely conventional and it is obvious that the symbols can be exchanged without influencing the derivation in the least. We also shall employ a different derivation than was used by Warburg (1924) because, to our minds, the derivation below is easier to understand and serves to emphasize that the derivation employed by Warburg is perfectly general; in short, that it is applicable to situations other than those described by him.

Taking first the flask with the smaller volume of fluid, the change observed on the manometer (h) is due to two things:

(a) The uptake of oxygen (h_{O_2})

(b) The release of CO_2 (h_{CO_2})

Thus:

$$h_{(observed)} = h_{O_2} + h_{CO_2}$$

but by definition:

$$h_{O_2} = x_{O_2}/k_{O_2} \text{ and } h_{CO_2} = x_{CO_2}/k_{CO_2} \text{ (since } x_{O_2} = h_{O_2}k_{O_2})$$

Therefore:

$$(12)\quad h_{(observed)} = x_{O_2}/k_{O_2} + x_{CO_2}/k_{CO_2}$$

If the same reaction has occurred in the second flask containing the larger volume of fluid, the change observed on the manometer (H) will similarly be:

$$(13)\quad H = X_{O_2}/K_{O_2} + X_{CO_2}/K_{CO_2}$$

However if the identical reaction has occurred in each flask, the amount of oxygen taken up (x_{O_2}, X_{O_2}) in each case should be the same, and the amount of CO_2 released should be the same (X_{CO_2}, x_{CO_2}) hence:

$$(13a)\quad x_{O_2} = X_{O_2};\ \text{and}\ x_{CO_2} = X_{CO_2}$$

Thus one may substitute (13a) in either equation (12) or (13) yielding:

$$(14)\quad \text{from equation (12)}\ h = X_{O_2}/k_{O_2} + X_{CO_2}/k_{CO_2} \qquad \text{or}$$

$$(15)\quad \text{from equation (13)}\ H = x_{O_2}/K_{O_2} + x_{CO_2}/K_{CO_2}$$

DETERMINATION OF OXYGEN EXCHANGE

One can combine equations (12) and (13) in another way. Suppose that one solves equation (12) for x_{CO_2} in the following way:

$$\text{equation (12)}\ h = x_{O_2}/k_{O_2} + x_{CO_2}/k_{CO_2}\ \text{hence,}$$

$$x_{CO_2}/k_{CO_2} = h - x_{O_2}/k_{O_2}\quad \text{or}$$

$$(16)\quad x_{CO_2} = k_{CO_2}\,(h - x_{O_2}/k_{O_2})$$

From equation (13), in the same way one obtains

$$(17)\quad X_{CO_2} = K_{CO_2}\,(H - X_{O_2}/K_{O_2})$$

But as was shown in equation (13a) $x_{CO_2} = X_{CO_2}$, hence equations (16) and (17) are equal to one another, or

$$(18)\quad k_{CO_2}\,(h - x_{O_2}/k_{O_2}) = K_{CO_2}\,(H - X_{O_2}/K_{O_2})$$

But equation (13a) also showed that $X_{O_2} = x_{O_2}$, hence this can be substituted to yield equation (19):

$$(19)\quad k_{CO_2}(h - X_{O_2}/k_{O_2}) = K_{CO_2}(H - X_{O_2}/K_{O_2})$$

The thing we are interested in determining is X_{O_2} (or x_{O_2}, since both are equal), hence solving for X_{O_2} as follows:

Multiply out equation (19):

$$hk_{CO_2} - X_{O_2}k_{CO_2}/k_{O_2} = HK_{CO_2} - X_{O_2}K_{CO_2}/K_{O_2}$$

Transpose terms:

$$X_{O_2}K_{CO_2}/K_{O_2} - X_{O_2}k_{CO_2}/k_{O_2} = HK_{CO_2} - hk_{CO_2}$$

Take out X_{O_2}:

$$X_{O_2}(K_{CO_2}/K_{O_2} - k_{CO_2}/k_{O_2}) = HK_{CO_2} - hk_{CO_2}$$

$$(20)\quad x_{O_2} = X_{O_2} = \frac{HK_{CO_2} - hk_{CO_2}}{\frac{K_{CO_2}}{K_{O_2}} - \frac{k_{CO_2}}{k_{O_2}}}$$

Note: One will sometimes find this equation written with the capital and small letters interchanged (Dixon, 1943). It may be noted that this is exactly the same equation except that the sign of both numerator and denominator have been changed.

The oxygen taken up (x_{O_2} or X_{O_2}) depends on the reading of one flask (H) times its CO_2 constant (K_{CO_2}) less the reading of the other flask (h) times its CO_2 constant (k_{CO_2}) divided by a constant which is calculated from the ratio of the CO_2 and oxygen constants of the first flask less the ratio of the same constants for the second flask. Equation (20) may be modified to:

$$(21)\quad x_{O_2} = X_{O_2} = HK_{CO_2} - hk_{CO_2}/C_{(O_2)}$$

where $C_{(O_2)}$ = constant = $K_{CO_2}/K_{O_2} - k_{CO_2}/k_{O_2}$

Once this constant ($C_{(O_2)}$) has been calculated from given experimental conditions, the calculation of the oxygen uptake becomes relatively simple. Normally one prepares a table of this sort:

(1)	(2)	(3)	(4)	(5)	(6)
H	HK_{CO_2}	h	hk_{CO_2}	Difference HK_{CO_2}-hk_{CO_2}	$\mu l.O_2$
Observed, corrected for thermobarometer changes.	Multiply column (1) by its flask constant for CO_2.	Observed, corrected for thermobarometer changes.	Multiply column (3) by its flask constant for CO_2.	Subtract column (4) from column (2).	Divide column (5) by $C_{(O_2)}$.

In making such calculations one must be careful to retain the algebraic sign of each measurement, i.e., if the level of fluid in the manometer drops, -h is used. If it rises, +h. (See example below).

DETERMINATION OF CO_2 EXCHANGE

In an entirely similar manner one may use equations (12) and (13) to calculate the CO_2 exchange. Suppose that one solves equation (12) for x_{O_2} in the following way:

From equation (12) $h = x_{O_2}/h_{O_2} + x_{CO_2}/k_{CO_2}$ hence

$$(22)\quad x_{O_2} = (h - x_{CO_2}/k_{CO_2})k_{O_2}$$

From equation (13) in the same way one obtains

$$(23)\quad X_{O_2} = (H - X_{CO_2}/K_{CO_2})K_{O_2}$$

But $x_{O_2} = X_{O_2}$, from equation (13a), hence equations (22) and (23) are equal to one another, or:

$$(24)\quad (h - x_{CO_2}/k_{CO_2})k_{O_2} = (H - X_{CO_2}/K_{CO_2})K_{O_2}$$

Equation (13a) also showed that $x_{CO_2} = X_{CO_2}$; hence for x_{CO_2} we can substitute X_{CO_2}

$$(25)\quad (h - X_{CO_2}/k_{CO_2})k_{O_2} = (H - X_{CO_2}/K_{CO_2})K_{O_2}$$

Equation (25) may be solved for X_{CO_2} (the CO_2 liberation or uptake which we are trying to determine) as follows:

Multiply out (25): $hk_{O_2} - X_{CO_2}k_{O_2}/k_{CO_2} = HK_{O_2} - X_{CO_2}K_{O_2}/K_{CO_2}$

Transpose terms: $X_{CO_2}K_{O_2}/K_{CO_2} - X_{CO_2}k_{O_2}/k_{CO_2} = HK_{O_2} - hk_{O_2}$

Take out X_{CO_2}: $X_{CO_2}(K_{O_2}/K_{CO_2} - k_{O_2}/k_{CO_2}) = HK_{O_2} - hk_{O_2}$ hence,

$$(26)\quad x_{CO_2} = X_{CO_2} = (HK_{O_2} - hk_{O_2})/\left(\frac{K_{O_2}}{K_{CO_2}} - \frac{k_{O_2}}{k_{CO_2}}\right) = (HK_{O_2} - hk_{O_2})/C_{(CO_2)}$$

The carbon dioxide taken up (if X_{CO_2} = -) or given off (if X_{CO_2} = +) is equal to the change in reading on one flask times the oxygen constant of that flask, less the change of reading on the other flask times its oxygen constant, divided by a constant.

Once this constant:

$$C_{(CO_2)} = K_{O_2}/K_{CO_2} - k_{O_2}/k_{CO_2}$$

has been calculated from given experimental conditions, the calculation of the CO_2 exchange becomes relatively simple. A table such as that used for oxygen may be used:

(1)	(2)	(3)	(4)	(5)	(6)
H	HK_{O_2}	h	hk_{O_2}	Difference HK_{O_2}-hk_{O_2}	$\mu l.CO_2$
Observed, corrected for thermobarometer changes.	Multiply column (1) by its flask constant for O_2.	Observed, corrected for thermobarometer changes.	Multiply column (3) by its flask constant for O_2.	Subtract column (4) from column (2).	Divide column (5) by constant $C_{(CO_2)}$. If value in column is + CO_2 is liberated; if - CO_2 taken up.

SUMMARY

By using two flasks of approximately equal volume, one of which contains more fluid than the other, two equations were derived in the previous paragraphs which permit one to determine the oxygen uptake (or release) and the CO_2 production (or uptake). These equations are:

$$(21)\quad x_{O_2} = X_{O_2} = HK_{CO_2} - hk_{CO_2}/C_{(O_2)}$$

$$(26)\quad x_{CO_2} = X_{CO_2} = (HK_{O_2} - hk_{O_2})/C_{(CO_2)}$$

Where the $C_{(O_2)}$ and $C_{(CO_2)}$ represent constants calculable from the experimental set-up employed.

This procedure permits one to determine O_2 uptake and CO_2 production in the presence of adequate supplies of CO_2, and thus enables one, by a comparison with the results of the "Direct Methods" described in Chapters 1 and 2, to determine whether or not CO_2 does influence the rate or the course of the process involved.

THE EQUATION CONSTANTS

The constants employed in the equations (No. (21), (26)) for calculating the oxygen and carbon dioxide exchange were defined as follows:

$$C_{(O_2)} = K_{CO_2}/K_{O_2} - k_{CO_2}/k_{O_2}$$

$$C_{(CO_2)} = K_{O_2}/K_{CO_2} - k_{O_2}/k_{CO_2}$$

In Chapter 6 methods of determining and calculating the flask constants (k or K) are described. Here it may be noted that::

$$K_{CO_2}/K_{O_2} = 1\Big/\frac{K_{O_2}}{K_{CO_2}}$$

It is therefore convenient to have not only the oxygen and carbon dioxide constants of each flask recorded for various volumes, but also their ratio. (It is not true, however, that $C_{(CO_2)}$ is the reciprocal of $C_{(O_2)}$.)

EXAMPLES OF THE USE OF WARBURG'S "INDIRECT METHOD"

We will cite an experiment on algae to illustrate the use of the "Indirect Method". 1 ml. of a suspension of Chlorella cells (containing 0.1 ml. packed wet cells) was placed in each of two Warburg flasks (No. 5 total volume = 13.9 ml.; No. 7, total volume 13.3 ml.). In the first flask was placed 1 ml. of M/1000 phosphate buffer (pH 4.5) (flask No. 5, $k_{O_2} = 1.08$, $k_{CO_2} = 1.22$); in the second, 5 ml. of the same phosphate solution was added (flask No. 7, $K_{O_2} = 0.67$, $K_{CO_2} = 1.09$). No added CO_2 was supplied to the air. After equilibration at 28°, readings were taken at 5 minute intervals. The readings obtained (corrected for thermobarometer changes) were as follows:

	Flask No. 5 (h)	Flask No. 7 (H)
First 5 minutes	0	-4.5
Second 5 minutes	-2	-5.5
Third 5 minutes	-1	-7.0
Fourth 5 minutes	0	-3.5

Oxygen uptake calculations are shown in Table X; CO_2 exchange calculations are shown in Table XI.

TABLE X

Oxygen Exchange

	Flask 7		Flask 5				
Time	H(mm)	HK_{CO_2}	h(mm)	hk_{CO_2}	Difference	$\mu l.O_2$	Sum
5	-4.5	-4.9	0	0	-4.9	-9.8*	-9.8
10	-5.5	-6.0	-2	-2.4	-3.6	-7.2	-17.0
15	-7.0	-7.6	-1	-1.2	-6.4	-12.8	-29.8
20	-3.5	-3.8	0	0	-3.8	-7.6	-37.4

$K_{CO_2} = 1.09$ $k_{CO_2} = 1.22$

* $= -4.9/C_{(O_2)} = -4.9/.5 = -9.8$

$K_{CO_2}/K_{O_2} = 1.09/0.67 = 1.63$ $k_{CO_2}/k_{O_2} = 1.22/1.08 = 1.13$

$C_{(O_2)} = K_{CO_2}/K_{O_2} - k_{CO_2}/k_{O_2} = 1.63 - 1.13 = 0.50$

TABLE XI

Carbon Dioxide Exchange

	Flask 7		Flask 5				
Time	H	HK_{O_2}	h	hk_{O_2}	Difference	$\mu l.CO_2$	Sum
5	-4.5	-3.0	0	0	-3.0	+11.1*	+11.1
10	-5.5	-3.7	-2	-2.2	-1.5	+ 5.6	+16.7
15	-7.0	-4.7	-1	-1.1	-3.6	+13.3	+30.0
20	-3.5	-2.3	0	0	-2.3	+ 8.5	+38.5

$K_{O_2} = 0.67$ $k_{O_2} = 1.08$

$C_{(CO_2)} = K_{O_2}/K_{CO_2} - k_{O_2}/k_{CO_2} = 1/1.63 - 1/1.13 = 0.615 - 0.885 = -0.27$ (see Table X)

* i.e. $-3.0/-0.27 = +11.1$

The data thus obtained have been plotted in Fig. 9 from which it is apparent that the R. Q. is very close to 1, i.e., for every molecule of oxygen consumed, one molecule of CO_2 is liberated. The example has been chosen to show that even relatively small changes in volume of either gas can be estimated quite accurately. It also has been chosen to illustrate, that under the conditions employed (i.e., pH 4.5) it is not necessary to add CO_2 to the gas phase, in order to measure the respiration. In Fig. 9 we also have drawn in the oxygen uptake curve (broken line) obtained on the same algae when the CO_2 was absorbed by KOH (in the Warburg "Direct Method", Chapter 1). Comparison of the two curves shows that

the presence of CO_2 does have an effect in this case, since respiration in the presence of CO_2 is less than respiration in its absence.

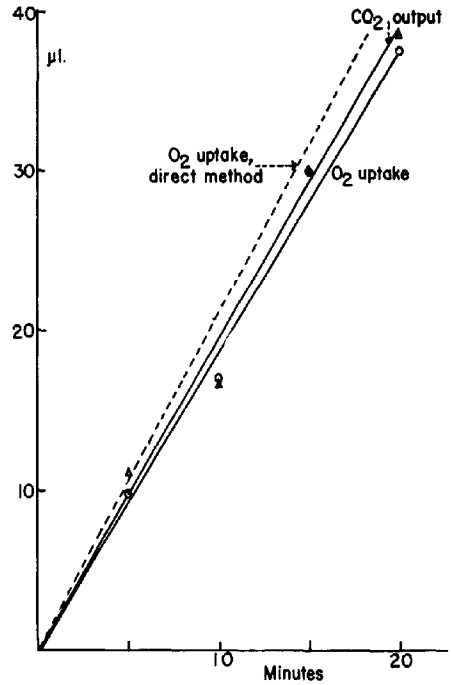

FIG. 9

Solid lines are graphs of data illustrating use of the "Indirect Method" of Warburg. The broken line is for oxygen uptake measured by the "Direct Method". See text.

A second example employing a slightly different principle follows. In this case flasks of different volumes containing the same volume of fluid are employed. The example below deals with an unsupplemented isotonic brain homogenate which one does not wish to dilute since soluble cofactors may diffuse out of the tissue. The reaction studied is the oxidation of glutamate in the presence of ammonia and adenylic acid, and for the sake of simplicity any effects of bound CO_2 and retention are ignored. In each of two flasks were placed the following materials: 0.2 ml. 0.1 M K_2HPO_4-KH_2PO_4, pH 7.3; 0.4 ml. 0.5 M KCl; 1.1 ml. isotonic (1.15%) KCl; 0.1 ml. 0.1 M $MgCO_3$; 0.2 ml. 0.1 M glutamate (pH 7); 0.1 ml. 0.1 M $(NH_4)_2HPO_4$; 0.3 ml. 0.01 M adenylic acid (pH 7); and 0.6 ml. isotonic KCl 10% rat brain homogenate. The larger flask (No. 12) had the constants $K_{O_2} = 1.54$, $K_{CO_2} = 1.70$, and the smaller flask (No. 1) had the constants $k_{O_2} = 1.06$, $k_{CO_2} = 1.22$. The readings obtained, corrected for thermobarometer changes, were as follows:

	Flask No. 1 (h)	Flask No. 2 (H)
1st 10 min.	-5	-2
2nd 10 min.	-10	-6
3rd 10 min.	-4	-2
next 20 min.	-7	-4

Calculations of oxygen uptake are shown in Table XII, and calculations of CO_2 exchange are shown in Table XIII.

TABLE XII

Oxygen Exchange

Time, Min.	Flask 12		Flask 1		Difference	µl. O_2	Sum
	H	HK_{CO_2}	h	hk_{CO_2}			
10	-2	-3.4	-5	-6.1	+2.7	-54*	-54
20	-6	-10.2	-10	-12.2	+2.0	-40	-94
30	-2	-3.4	-4	-4.9	+1.5	-30	-124
50	-4	-6.8	-7	-8.5	+1.7	-34	-158

$$C_{(O_2)} = \frac{1.70}{1.54} - \frac{1.22}{1.06} = 1.10 - 1.15 = -0.05$$

* $\frac{2.7}{-0.05} = -54$

TABLE XIII

Carbon Dioxide Exchange

Time, Min.	Flask 12		Flask 1		Difference	µl. CO_2	Sum
	H	HK_{O_2}	h	hk_{O_2}			
10	-2	-3.1	-5	-5.3	+2.2	+55	55
20	-6	-9.3	-10	-10.6	+1.3	+33	88
30	-2	-3.1	-4	-4.2	+1.1	+27	115
50	-4	-6.2	-7	-7.4	+1.2	+30	145

$$C_{(CO_2)} = \frac{1.54}{1.70} - \frac{1.06}{1.22} = 0.91 - 0.87 = +0.04$$

The results have been plotted in Fig. 10 together with the oxygen uptake results obtained by means of the direct method (Chapter 1) and the carbon dioxide results obtained by the direct method (Chapter 2). Note first that while there are differences (the indirect method gave higher values) there is also relative agreement. This is striking if one considers that the actual readings in the case of the indirect method were from 2 to 5 mm. per interval. Yet the results obtained are a reasonably accurate estimate of the progress of the reaction.

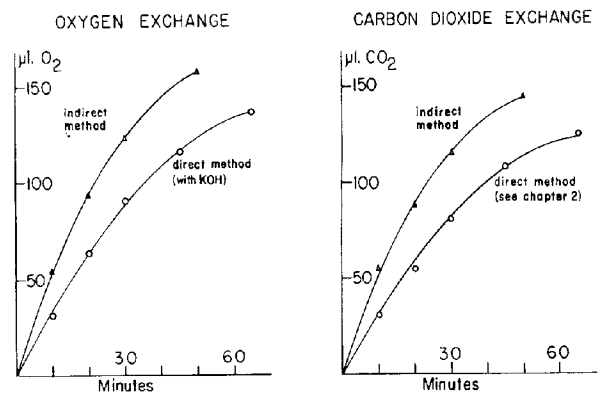

FIG. 10

Graphs of data obtained for oxygen exchange and carbon dioxide exchange by the direct and indirect method.

APPLICATION TO OTHER GASES

It should be pointed out that this type of system can be applied to gases other than CO_2 and O_2. For gases "a" and "b", the equations (No. (20), (26)) become

$$x_a = X_a = \frac{HK_b - hk_b}{\frac{K_b}{K_a} - \frac{k_b}{k_a}} \qquad \text{and } x_b = X_b = \frac{HK_a - hk_a}{\frac{K_a}{K_b} - \frac{k_a}{K_b}}$$

One may note that should gases "a" and "b" have the same solubility, then $K_a = K_b$ and $k_a = k_b$ hence both equations equal infinity. Hence one can only apply this method to gases whose solubilities differ appreciably.

PRACTICAL USE OF INDIRECT METHODS

In order to obtain data upon which a calculation may be based, one can do any of three things:

1. Using flasks of approximately the same volume, employ markedly different volumes of fluid.

2. Using the same volume of fluid, employ flasks of different volume. (See, for example, Emerson and Lewis, 1941 and example above.)

3. Using flasks of the same volume, employ different amounts of fluid, and markedly different amounts of tissue such that the ratio of tissue to fluid medium is the same in both cases. The derivations of this third type of use are somewhat different than previously described. Since we have not used this procedure, and since it offers no advantages over the two procedures listed above, we omit consideration of it here. Essentially one holds the tissue to fluid medium ratio constant, divides the observed manometer changes by the amount of tissue, and then calculates as described in the examples given. We have yet to convince ourselves that this is a justifiable procedure, but Dixon (1943, page 77) considers it "preferable" to procedure 2 (above).

For a given situation, not all of these methods are equally suitable. For example, a reaction whose rate is dependent upon the concentration of a diffusible substance, such as a coenzyme, will not proceed at the same rate in a flask containing a large amount of liquid as in a flask containing a small amount of liquid. Hence, procedure 1 will give erroneous results, whereas procedure 2 will give valid results. Under other circumstances procedure 1 might be preferrable to procedure 2.

The derivations we have described above are somewhat different from those given by Warburg (1924). They have been developed in this way to emphasize the broad applicability of this method. Warburg's treatment, while a perfectly general one, has been more or less interpreted as a specific solution to the problem, and workers using the method have usually attempted to duplicate the conditions used by Warburg, rather than to employ the principles he emphasized in methods more suited to their conditions. It is not necessary for the success of this method to use the modified flasks Warburg describes, to work in bicarbonate buffers at the concentration and at the pH that he employs, nor is it necessary to work in an atmosphere of 5% CO_2. These conditions may be altered by the investigator at will and by the application of the laws of CO_2 - bicarbonate - pH equilibria, outlined in Chapter 3, a wide range of conditions may be employed.

ADAPTATION TO MORE COMPLEX MEASUREMENTS

In the previous derivations and discussion we have described how it is possible to determine two gases simultaneously if their solubilities differ. One would have a very reasonable chance of determining oxygen and hydrogen, for example. Physiologically, however, we are interested mostly in oxygen and carbon dioxide, and, as discussed in Chapter 3, carbon dioxide while obeying the general laws of solubility, also forms carbonic acid which can react with bases, and this alters the entire "solubility" picture.

If one is studying a reaction which produces no acid, but only CO_2, and can operate at a pH below 5 (for example, urease acting on urea) one may employ a system with no CO_2 added to the atmosphere. Such reactions are, however, quite rare. An example of oxygen uptake and CO_2 release under these circumstances is the respiration of acid tolerant sulfur bacteria (Vogler, LePage and Umbreit, 1941), but with few exceptions (Vogler, 1942) such reactions are not sensitive to the presence of CO_2 and can thus be more readily measured by more direct methods.

In the more normal physiological ranges, bicarbonate buffers may be employed; in this case a CO_2 pressure in the atmosphere is required (see Chapter 3) in order to maintain a given pH. If the concentration of bicarbonate ion is at least 10 times greater than any

other carbon dioxide retaining agent, the practical error involved from CO_2 retention by other materials is usually negligible. Thus it is possible to use a Ringer's solution with bicarbonate at a definite CO_2 pressure and obtain adequate measurements of gas exchange. It is under these circumstances that the method has been most widely used. As discussed previously, another difficulty arises if acid is produced as well as CO_2, since both will appear in the pressure changes as CO_2. What has usually been done is to assume a definite and constant R. Q. (usually taken as 0.9 or 1.0), i.e., for every oxygen taken up, one (or 0.9) CO_2 is released. One then takes any extra CO_2 beyond this figure as being due to acid production. But frequently, far from permitting assumptions of a definite and constant R. Q., this has been just the point which one wished to measure. Actually the problem of distinguishing between "respiratory CO_2" and acid production under these circumstances has never been critically solved although ingenious methods which approach a solution have been devised (Dixon, 1943).

If one wishes to work with media containing large amounts of CO_2-binding materials (protein, serum, etc.), the retention of CO_2 in the medium may become so large as to entirely invalidate the results of such measurements. The problem of "serum retention" (Warburg, 1925) has been approached and a reasonably satisfactory solution supplied for a few cases. But so complex do the conditions of operation become that most workers have studiously avoided such experiments. It is beyond the scope of this manual to describe these methods since they are admittedly too advanced for the beginner and are, in addition, of rather limited application. Before undertaking such studies a relatively long experience with manometric methods is probably necessary. Descriptions of the techniques employed and the theory upon which they are based will be found in papers by Warburg (1925), Warburg (1926), Warburg, Kubowitz, and Christian (1931), Dixon (1943), Dixon and Elliott (1930). The most general use of the "indirect" methods of Warburg has been in the determination of whether the presence of CO_2 (as bicarbonate) does indeed influence the reactions one is studying. If CO_2 is without effect, the "direct" methods are convenient and generally preferable. A short discussion of "retention" will be found in Chapter 8.

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Chapter V

USEFUL TECHNIQUES IN MANOMETRY

The practical operation of any instrument always involves details which are not readily recognized by the description of the procedures or the theory of the instrument. We have collected below some of the techniques which are useful with the Warburg instrument.

CLEANING GLASSWARE

All reaction flasks must be cleaned thoroughly. There are many methods for cleaning manometric glassware, but only a few which are widely used will be described here. The most suitable method to employ will depend upon the reactions and types of enzyme preparations being studied. Often tissue homogenates are sensitive to metal contamination, whereas bacterial suspensions may be insensitive to small amounts of metals but markedly affected by traces of vitamins or other organic substances. When fatty preparations are used, thorough washing with an organic solvent is necessary; if the film of fat is not removed it may permit alkali to "creep" out of the center well. Under many conditions a hot water rinse is adequate for removing grease. Three practical methods for cleaning reaction vessels follow:

1. Dichromate method:
   (a) Place flasks in unleaded gasoline to remove grease, or remove with gasoline on a cotton swab.
   (b) Wash with water.
   (c) Place in cleaning solution for 12 - 24 hours. (Cleaning solution: dissolve 63 g. sodium (or potassium) dichromate by heating with 35 ml. water. Add conc. H_2SO_4 to 1 liter.)
   (d) Remove, wash by rinsing in distilled water at least six times. Some operators place the flask in dilute NaOH (5 g./liter) after removing the excess chromic acid before rinsing with distilled water.

2. Nitric acid method:
   (a) Place flasks in gasoline for 30 minutes to remove grease, or remove with gasoline on a cotton swab.
   (b) Wash with water.
   (c) Transfer to a mixture of equal parts of concentrated H_2SO_4 and HNO_3 in a Pyrex dish. Heat this mixture for 30 to 60 minutes in a hood. Cool.
   (d) Remove, wash several times in distilled water.

3. Calgon method:
   (a) Remove grease with gasoline on a cotton swab. Rinse with water.
   (b) Immerse in a pan containing 1 tablespoonful Calgon per gallon of water. (Other detergents comparable to Calgon may be employed; sulfonated higher alcohols also have been used successfully.) Boil gently for 15 minutes. Excessively long heating or boiling the pan dry will accelerate etching of the flasks. A cylindrical Pyrex jar with a copper coil immersed in the Calgon solution provides a convenient cleaning bath; the bath is heated by passing steam through the coil. The immersed flasks also may be heated in the autoclave.
   (c) Rinse several times with distilled water.

OPERATIONAL TECHNIQUES

Grease: The grease used in lubricating the ground glass joints of the flask-manometer connections or the plugs for the sidearms is usually either anhydrous lanolin or heavy vaseline. The grease used for the stopcock on the manometer is preferably a good stopcock grease such as is used for burettes.

At high temperatures Celevacene light (Distillation Products Co.) is useful because of its small change in consistency with changing temperature. The use of silicone greases is not advised because they are very difficult to remove from glassware.

Brodie's Solution: A formula of a convenient solution is the following:

23 grams NaCl
5 grams Sodium cholate (Merck)
in 500 ml. water
Density 1.033
P_o = 10000

Evan Blue (200 mg./liter) or acid fuchsin are excellent dyes for the fluid; other dyes may be used, but some of these tend to decompose in the manometer. Obviously many materials may be used in the manometers, and for each it is only necessary to know its density.

Organic Liquids as Manometer Fluids: A possible objection to the use of Brodie's solution, although not a particularly serious one, is that the evaporation of water from the solution in the manometer increases the salt concentration and density of the manometer fluid. A single compound with a density equal to that of Brodie's fluid would provide a manometer fluid that would not change in density upon evaporation. An inspection of tables of organic compounds reveals a considerable number with densities close to 1.033 g./ml. (P_o = 10,000). Many of these are of little utility because they are difficult to obtain, expensive, volatile, too viscous, or because they attack rubber. Ethyl lactate (1.031 g./ml. at 20°C.), plus crystal violet or malachite green for coloring, is the best of the compounds we have tried; it is somewhat more sluggish in its response than is Brodie's solution.

Clerici Solution:

7 grams thallium formate
7 grams thallium malonate
1 ml. water
Density about 4; P_o value about 2500

Mercury: It is convenient, when using mercury, to place a drop of water at the top of each column. This permits the mercury to flow freely. Density about 13.6 (dependent on temperature); P_o = 760.

Removing bubbles in manometer column: If the column of Brodie's fluid is broken in the manometer it may be readily joined again by rapidly compressing the rubber Brodie fluid reservoir with the finger and then releasing the pressure slowly. Repeating this soon raises the bubbles to the surface of the liquid.

Adding manometer fluid: Draw the fluid into a hypodermic syringe. Jab the needle through the rubber tubing near the base of the reservoir and inject the desired quantity of fluid. This is a good method also for adding fluid to or removing it from a manometer. The rubber tubing reservoir may be filled before it is attached to the manometer and additions of fluid can be made through the open arm of the manometer (if the column of fluid is broken in the process it may be rejoined as described in the preceding paragraph).

W. W. Umbreit, R. H. Burris, J. F. Stauffer

READING THE MANOMETER AFTER IT HAS PASSED THE GRADUATED RANGE

It sometimes happens that, due to the addition of gases or their absorption, the adjustment of the fluid in the closed arm of the manometer causes the fluid in the open arm to come to rest at a point off the graduated scale of the open arm. A method (Vogler, 1942) for adjusting the zero point on the closed arm so that a reading can be obtained follows:

Adjust the fluid in the closed arm until that in the open arm is on the scale. The distance one has moved the fluid in the closed arm from the zero point is called "e". Record the reading in the open arm. Adjust the closed arm so that the fluid is a distance 2e from the zero point. Record the reading in the open arm. The difference between the two readings of the open arm is the amount to be added to or subtracted from the first reading to give the actual reading if the closed arm had been at the zero point.

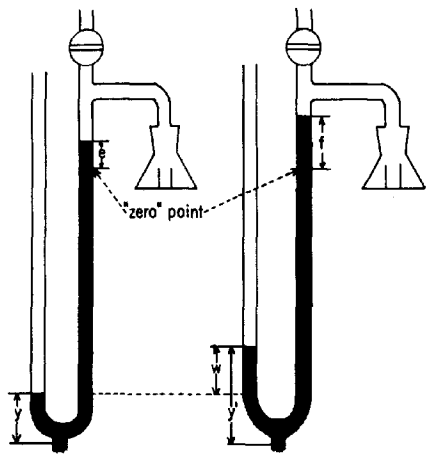

FIG. 11

Diagram illustrating the method of reading the manometer after it has passed the graduated range.

Theoretical: If the volume of gas (V_g) be decreased x by raising the level of manometer fluid in the closed arm by e cm., the corresponding pressure P_o will be increased by y so that:

$$(V_g - x)(P_o + y) = V_g P_o \quad \text{or}$$

$$V_g y = x (y + P_o) \quad \text{(See Fig. 11).}$$

Similarly if the fluid in the closed arm be raised f cm., the corresponding volume change (x') will cause an increased y' so that:

$$V_g y' = x'(y' + P_o)$$

Thus:

$$y/y' = x (y + P_o)/x'(y' + P_o)$$

Since $x = \pi r^2 e$ and $x' = \pi r^2 f$, where "r" is radius of the capillary tube of the manometer:

$$y/y' = \pi r^2 e (y + P_o)/ \pi r^2 f (y' + P_o)$$
$$= e (y + P_o)/f (y' + P_o)$$

Since P_o is large (10,000 mm. of Brodie's solution), and y and y' are small, an accuracy of 1% is possible, if y and y' are not greater than 100 mm. (10 cm.), by considering $y + P_o = y' + P_o$, from which $y/y' = e/f$.

If W were defined as the difference between the reading at y and y' (closed arm at e or f), then

$$y' - y = W \quad \text{or} \quad y' = W + y$$

Thus:

$$y = \frac{y'e}{f} = \frac{(W + y)\, e}{f} = \frac{We + ye}{f}$$

$$fy = We + ye$$

$$y (f - e) = We$$

$$y = \frac{We}{f-e}$$

If f were chosen to equal 2e, then y = W.

"y" is the distance off the scale (when closed arm is at zero point) from the first reading on the scale (when closed arm is at e).

ILLUSTRATION 1. Oxygen uptake is unexpectedly rapid and upon returning the closed arm to its zero point (250 mm.) the open end does not reach zero. By adjusting the closed arm to 260; the reading is 3; by adjusting to 270, the reading is 8. Hence e = 10; f = 20; W = 5; y = We/f - e = 5. Reading at zero point was 3 - 5 = -2.

ILLUSTRATION 2. In order to obtain a reading on the scale, it was necessary to adjust the closed arm to 280; (e = 30 from zero point of 250) hence 2e is impossible (310), since closed arm scale will not read to this point. Reading at 280 = 5. Reading at 300 = 16.

$$y = We/f - e = 11 \times 30/50 - 30 = 330/20 = 16.5$$

Reading at zero point = 5 - 16.5 = -11.5.

W. W. Umbreit

ANAEROBIC MEASUREMENTS

Anaerobic conditions are generally obtained in respiration vessels by thorough flushing with nitrogen freed of oxygen. The oxygen-free nitrogen is passed directly from a tank or from a combustion tube through a manifold to the Warburg flasks which are shaking in the bath. Ten minutes of slow flushing or passage of a liter of gas through each flask should suffice to remove oxygen. Linde Air Products now supplies high purity dry nitrogen with a minimum purity of 99.99%. This gas is inexpensive, and a tank of it supplies by far the most convenient source of gas for producing anaerobic conditions. If such a source of nitrogen is not available, ordinary tank nitrogen may be purified by passing it through heated copper turnings in a combustion tube at 600 to 700°C. A more thorough removal of oxygen is accomplished by passing gas through cupric hydroxide which has been precipitated on diatomaceous earth and then dried and reduced with hydrogen. This mixture is effective at 200 to 300°C. (Meyer and Ronge, 1939). Other gases, e.g. hydrogen or helium, may be purified by the same procedures as nitrogen (with hydrogen the combustion train must be flushed to remove oxygen before the heat is applied).

The evacuation procedure described under "Altering Gas Mixtures" may be used to obtain anaerobic conditions. Four evacuations to 75 mm. Hg residual pressure should leave only about 0.3 µl. of the original oxygen in a 15 ml. flask. The gas may be added directly from a tank or from a heated combustion tube.

Alkaline pyrogallol in an absorption tower is less effective than hot copper for removing contaminating oxygen. In addition, alkaline pyrogallol absorbs any CO_2 present in the gas mixture, and certain concentrations of alkaline pyrogallol are reported to liberate CO. Small pieces of freshly cut yellow phosphorus added to the center well or side-bulb of a Warburg flask will remove residual oxygen that may remain after incomplete flushing. However, a thorough flushing with oxygen-free gas should obviate the need for this. The addition of phosphorus is somewhat undesirable, as the P_2O_5 fumes which form before oxygen-free gas is passed through the system may be absorbed with resultant alteration in the pH of the medium. When phosphorus is depended on to rid the flask of oxygen, initiation of the reaction must be delayed until the manometer indicates oxygen uptake by the phosphorus has ceased.

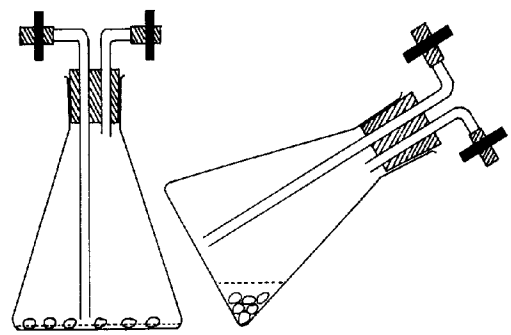

FIG. 12

Flasks for producing oxygen-free gases.

Occasionally the use of hot copper for removing oxygen may be unde-

sirable, for example, Keilin and Hartree (1943) found that nitrous oxides formed in the reaction inhibited the action of catalase. The following method will serve under such circumstances: To a stoppered Erlenmeyer flask or bottle (as indicated in Fig. 12) add 1 to 2 grams of yellow phosphorus and sufficient water to cover the phosphorus when tilted but not sufficient to cover the phosphorus when the flask is level. Fill the flask with the gas mixture (preferrably by evacuation; see below), and then expose the phosphorus by placing the flask in a level position. Leave it in this manner for 2 to 3 days. The treatment may be shortened to 20 to 30 minutes by placing the flask in a water bath at 60°C. (the phosphorus melts and must be shaken frequently). The phosphorus reacts with any oxygen present to produce phosphorus pentoxide which gradually dissolves in the water and forms phosporic acids. The oxygen-free gas then may be transferred to Warburg flasks by the evacuation method described below. Suction flasks are desirable when the gas mixtures are prepared by evacuation. With explosive gas mixtures (e.g., those containing H_2) exposure of the phosphorus should be made slowly and the operator should be protected.

ALTERING GAS ATMOSPHERES

At times it is necessary to work with gas mixtures other than air. For this purpose sidearm flasks of the form shown in Fig. 13 are used; when the stopper of the sidearm is appropriately turned gas can be vented through the sidearm. Gas mixtures are supplied through the opened stopcock of the closable arm of the manometer.

FIG. 13

Sidearm flask with gas-vent.

It is necessary to run about a liter of gas through the flask to assure that all air has been displaced. By the use of a manifold arrangement an entire bank of flasks may be supplied with gas at one time. As an indication of the rate of gas flow a "U tube" is attached with rubber tubing to the sidearm stopper. The end of the glass "U" is pulled out into a capillary and dips below the surface of the water in the constant temperature bath; bubbles from the tube show the passage of gas.

When a uniform gas mixture is to be used routinely, e.g., 5% CO_2 95% O_2, it is convenient to purchase such a mixture in steel cylinders. When small quantities of a number of mixtures are necessary they may be prepared in stoppered bottles by filling the bottles with water and displacing measured quantities of water with the gases comprising the mixture--the water is displaced directly into a graduated cylinder for estimation of volume. Alternatively, the stoppered bottle may be evacuated, flushed with one of the component gases and then filled with the various gases to the desired pressures as indicated by a mercury manometer. The gas mixtures may be displaced from the bottles with water and passed through the Warburg flasks. (When the mixtures contain CO_2, the use of water as a displacing fluid will alter the composition of the gas mixtures unless the water is previously saturated with CO_2 at the CO_2 pressure used.)

Displacing air from respiration flasks by flushing with a flowing stream requires considerable amounts of gas. This procedure is costly with expensive gases and undesirable when carbon monoxide or other poisonous gases are being used. To decrease the amount of gas necessary to flush a flask we have employed an evacuation procedure. With the McGilvery manometer (Chapter 8) it is particularly convenient to gas by evacuation; with the Warburg manometer the following method is applicable: Tubes in the form of a reversed h with a T at the top of the h are connected in series (a bank of 7 manometers is easily treated at one time), as illustrated in Fig. 14, and attached with rubber tubing to both arms of the Warburg manometer. The Brodie fluid is lowered to within a few cm. of the bottom of the manometer columns, as it will rise during the evacuation. After making sure that the manometer stopcocks are open, the screw clamp A is opened and the system is evacuated by means of a water aspirator until there is about 75 mm. Hg residual pressure, as indicated by mercury manometer C. Screw clamp A is now closed and clamp B opened; water in bottle D displaces gas from storage bottle E into the system until atmospheric pressure is reached. After two more evacuations and refillings the replacement of the original atmosphere is sufficiently complete, i.e., approximately 0.1% of the original

gas remains. On the last filling the pressure is allowed to build up until gas spills through the mercury manometer. The rubber tubing is then removed at F and the excess gas is lost from the flasks obviating the difficulties that would arise if a small vacuum were left to suck air into the flasks. The stopcocks on the manometers are closed immediately and the manometers placed on the bath; as the flasks warm up the stopcocks must be opened momentarily to release gas. If desired, the evacuation procedure may be performed in the constant temperature bath, though this generally presents no advantage.

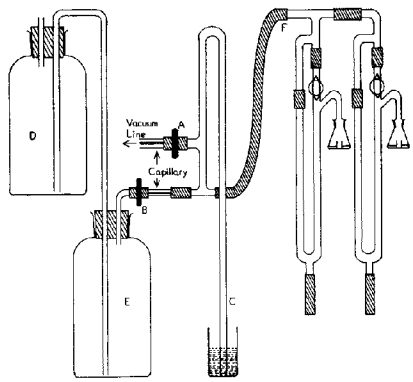

FIG. 14

Apparatus for altering the gas atmosphere by the evacuation method.

When the system is under vacuum, the rubber tubing which serves as a reservoir for Brodie fluid will be compressed pushing the fluid up in the manometers. It is necessary to use tubing with reasonably heavy walls to minimize this effect. Although a vacuum better than 75 mm. Hg residual pressure can be obtained readily, its use is not recommended routinely because of the bubbling of the Brodie fluid that may occur. It is helpful to de-gas the Brodie fluid under vacuum before filling the manometers. Capillaries are introduced in the vacuum and gas lines to limit the rate of gas flow; sudden changes in pressure are not registered uniformly on the two columns of the Warburg manometer because the large volume of gas leaving or entering the Warburg flask must pass through the capillary tubing of the manometer. If the stopcock of the manometer is closed or plugged, vacuum is applied to one side of the manometer only, and fluid may be displaced into the evacuation line.

The method of adding gases by evacuation requires only a tenth to a twentieth as much gas as the flushing procedure, and one is always certain that every dead space in the most complicated flask has had its initial gas displaced by the desired gas mixture. Although the procedure was evolved originally to save valuable gases it has proved so convenient that we use it routinely.

R. H. Burris

METHODS OF PREPARING GAS MIXTURES

Displacement Procedures: If pure gases are available, mixtures may be prepared by the displacement of water or other fluid. One usually prepares flasks or bottles completely filled with the confining fluid, and allows the various gases to enter until a given quantity of the fluid has been displaced. The examples below will clarify the actual procedure:

Example: Gas mixture required: 100 ml. of 20% O_2, 30% H_2, 50% N_2. Pure N_2 is added to displace 50 ml. of water from a flask completely filled with water. 20 ml. of water are displaced with O_2 and a further 30 ml. displaced with H_2. An alternative procedure, in case pure N_2 were not available would be: Add 50/.8 = 62.5 ml. air (of which 50 ml. is N_2, 12.5 ml. O_2), 7.5 ml. O_2 (20 ml. required in all less 12.5 ml. added with air equals 7.5 ml. still required), and 30 ml. H_2.

Displacement procedures are quite adequate, but one must be certain that the gas displacing the water is at atmospheric pressure, or at least that all gas added to form the mixture is supplied at the same pressure.

Evacuation Procedure: Atmospheric pressure is observed on a barometer. The gas bottle, in which the mixture is to be made, is attached to a manometer, as shown in Fig. 15. Connection A is closed (and kept closed throughout the operation), B is opened, and the flask is evacuated by attaching a water pump at C. Gases are added through C until the proper mixture is obtained. The procedure is best illustrated in the examples below. Any shaped container of any volume may be employed with no alteration in the method of preparing the gas mixtures.

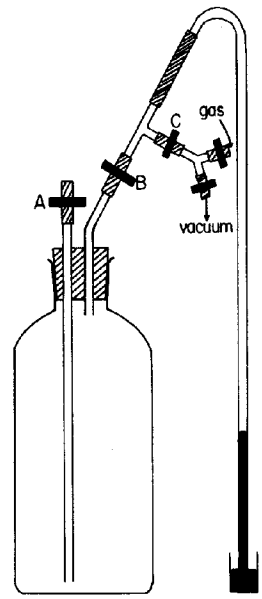

FIG. 15

Apparatus for preparing gas mixtures by evacuation and refilling.

Example 1: Gas mixture required: 20% O_2, 30% H_2, 50% N_2, Atmos. pressure = 748 mm. Hg. Using air for the nitrogen source (assuming 80% N_2 and 20% O_2 in air), one needs 50% of 748 mm. = 374 mm. of N_2. To obtain this amount of N_2 from air, one needs 374/.8 = 467.5 mm. of air. Hence one would evacuate with the water aspirator until the mercury in the manometer stood at (748 - 467.5 =) 280.5 mm. 20% oxygen requires 20% of 748 mm. = 149.6 mm. However 93.5 mm. have already been added with the air, hence 56.1 mm. of pure oxygen are to be added. One then attaches an oxygen source to C, and admits oxygen until the level of mercury in the manometer reaches (280.5 - 56.1 =) 224.4. 30% hydrogen requires 30% x 748 mm. = 224.4 mm., hence one attaches a hydrogen source to C, and permits hydrogen to enter until the manometer reaches zero (224.4 - 224.4 mm.).

To prepare gas mixtures which contain no oxygen, evacuate the gas bottle, refill with one of the components of the gas mixture, repeat this process twice, and proceed from this point. This is illustrated in the two examples given below:

Example 1: Gas mixture required: 80% H_2, 5% CO_2, 15% N_2. Atmospheric pressure, 748 mm.; aspirator can evacuate to 720 mm. Evacuate to 720, return to zero with H_2; evacuate to 720, return to zero with H_2. Evacuate to 149.6 mm. (gas remaining in bottle is H_2); add CO_2 to 112.2 (from 149.6 - (0.05 x 748)), return to zero with pure N_2.

Example 2: Gas mixture required: 20% H_2, 20% N_2, 60% He. Atmospheric pressure, 748; aspirator can evacuate to 720 mm. Evacuate to 720, return to zero with H_2 or N_2 (helium is more expensive and is not employed in the flushing out process). Repeat twice. Evacuate to 598.4 (gas remaining in bottle is H_2 or N_2), return to 448.8 with N_2 (or H_2); return to zero with Helium.

THE USE OF CYANIDE AS AN INHIBITOR

Cyanide is a useful inhibitor in the study of oxidations because it penetrates cells readily and strongly inhibits heavy metal (not necessarily iron) catalysts. HCN is a weak acid and its salts in solution consist largely of undissociated HCN. Further, in spite of its ready solubility, HCN is volatile and is absorbed, along with CO_2, by the alkali. Similar properties have been reported for azide (Machlis, 1944). In the previous edition of this book a theoretical analysis of HCN volatility was given together with tables of KOH-KCN mixtures for CO_2 absorption in the center alkali cup. The composition of these mixtures, essentially as described by Krebs (1935), was intended to be such that HCN would be in equilibrium between the alkali well and the reaction chamber so no interchange of HCN would occur. However, Riggs (1945) has demonstrated that the assumptions made are not valid and that experimentally a much more complex situation is involved. He suggests, therefore, that the cyanide concentration in the experimental vessels should be determined empirically. However, Robbie (1946, 1948) and Robbie and Leinfelder (1945) have developed methods for maintaining the cyanide concentration in the experimental fluid at known levels. The best of these methods involves the use of mixtures of calcium cyanide and calcium hydroxide. These mixtures are applicable to a wide range of concentrations and provide a constant tension of HCN (and a virtually constant pH in the center well) even though gas is given off or CO_2 absorbed. Since $Ca(OH)_2$ is only slightly soluble and a

large excess is kept in suspension in the center well, a reserve is constantly available to replace that lost by precipitation of $CaCO_3$. In addition the very large reserve of HCN in the center well permits the adjustment of the center well concentrations so that they will provide the HCN necessary for the experimental fluid; thus this fluid will differ from a control only by the presence of HCN.

TABLE XIV

Composition of Center Well Fluid at 37.5° C.*

HCN	$Ca(CN)_2$	KCN
10^{-2} M	1.45 M	-
10^{-3} M	0.32 M	-
10^{-4} M	0.38 M	0.83 M
10^{-5} M	0.0046 M	0.078 M
10^{-6} M	0.0005 M	-

*Adapted from Robbie (1948). Calcium cyanide solutions contain 10% $Ca(OH)_2$ suspension. KCN solutions contain 0.5 M KOH. For intermediate concentrations and for other temperatures refer to Robbie (1948).

The methods of preparing the calcium cyanide and its use are described in detail by Robbie. For convenience a few of the data are collected in Table XIV, but before precise work is done on cyanide inhibition the publications of Robbie, particularly Robbie (1948), should be consulted.

ADDITION OF MATERIALS DURING THE COURSE OF THE REACTION

It frequently is desirable to add substances to reaction flasks during the course of the reaction. This usually is accomplished by using Warburg flasks equipped with one or more sidearms (Fig. 16). After tipping in material from a sidearm, rinse the sidearm with some of the fluid from the main compartment of the flask, and then tip this back into the main compartment. Without such a rinse quantitative transfer of the material from the sidearm is not obtained. When removing manometers and attached flasks from baths for such operations, place a finger over the open end of the manometer; this prevents expansion or contraction arising from temperature changes from pushing out or sucking back the fluid from the manometers. Do not fill sidearms to full capacity. If the sidearm is capable of holding 1 ml., it is best employed for contents of 0.5 ml. or less. This not only prevents any material from spilling over into the main compartment prematurely, but also allows more rapid attainment of equilibrium between the gas phase and the liquid in the sidearm. Unless the two phases are at equilibrium, gas may suddenly be taken up or evolved when the material from the sidearm is tipped into the main compartment.

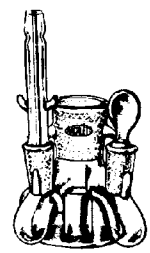

FIG. 16

Double sidearm Warburg flask.

It also is well to adjust the composition of the materials in the sidearm so that only one factor is altered upon the addition of its contents to the main compartment. For example, addition of 0.5 ml. M/10 glucose to a bacterial suspension in M/50 phosphate buffer alters not only the glucose concentration but the phosphate concentration as well.

"Keilin cups" provide a means for adding materials to flasks with insufficient sidearms. "Keilin cups" are small tubes (Keilin, 1929) provided with a small hook, of platinum wire or glass, by which they may be hung from the edge of the center well. They can

be dislodged by a careful jarring of the apparatus. It is possible (as pointed out by Dixon, 1943) to add more than one material by use of two "Keilin cups" with hooks of different lengths; one cup is released by a less vigorous jar of the apparatus than the other. Usually, however, the addition from sidearms is preferrable. Among the most useful "all purpose" flasks are the two sidearm flasks equipped with a gas venting plug (Fig. 16).

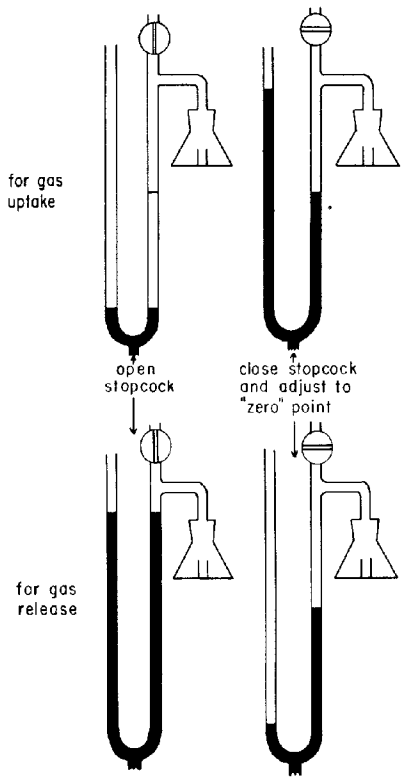

FIG. 17

Diagram illustrating the manometer setting to obtain maximum range.

ZERO POINTS

The zero point should be chosen to make maximum use of the graduated scale of the manometer. When gas uptakes are measured 250 mm. is a convenient point; when gas evolution is measured 50 mm. is convenient.

The following method using 150 mm. as the zero point is suggested by Dr. Cohen: In measuring gas uptake the manometer fluid is set, with the stopcock open, near the bottom of the scale as shown in Fig. 17. The stopcock is closed. Adjusting the fluid to the zero point of 150 mm. in the closed arm raises the fluid in the open arm to near the top of the graduated scale. In measuring gas release, the opposite type of setting is employed as indicated in Fig. 17. This permits the use of the same zero point for both uptake and release of gas and makes maximum use of the graduated scale.

W. W. Umbreit

CONSTANT TEMPERATURE BATHS AND SHAKING APPARATUS

The basic requirements for the bath which holds the microrespirometers are that it be capable of maintaining a uniform constant temperature throughout and of providing adequate shaking of the flasks. Wide latitude in shape and size of the bath is permissible, but in the past the standard design has been a rectangular bath carrying 7 manometers on each of 2 sides. Shaking has been provided by a rocking or a reciprocating motion.

Recently, Lardy, Gilson, Hipple and Burris (1948) have described in detail a circular bath and a modified shaking mechanism which possess distinct advantages over the earlier apparatus (Fig. 18; photograph courtesy GME, 4 Franklin St., Madison, Wisconsin. A circular bath with pivotal shaking is also sold by American Instrument Company). Manometers may be placed around the entire periphery of a circular bath, so the unit is more compact than a rectangular bath of equal capacity. A bath 22 inches in diameter will accommodate 18 manometers. The extra manometers available substantially improve the productivity of the apparatus. The instrument is arranged so that the entire shaking mechanism may be rotated, thus each manometer may be brought directly in front of the operator without his changing position. This feature permits the apparatus to be installed in a corner or along a wall with only one side of the bath accessible, whereas the rectangular bath must occupy a space-consuming, open position in the room to provide accessibility from 3 sides. A uniform temperature is easily maintained in the circular bath, for it contains no "dead spots" to hinder circulation. The water is circulated either with a centrifugal pump mounted beneath the bath or with a stirring motor mounted on the central column.

The first circular models were provided with the conventional rocking type of shaking device. This was chosen because the manometers can be read without stopping them, whereas a reciprocating shaker must be stopped for accurate reading because the manometer fluid bounces excessively. Later circular models have employed a pivotal shaker; the flask describes a considerable arc while the manometer scales move very little. The slight movement of the manometer scales is toward and away from the observer, and the scales may be read while moving with virtually the same ease as when they are stationary. Comparisons of the rocking, reciprocating and pivotal types of shaker have shown them all to be very effective in providing agitation of the contents of the flasks.

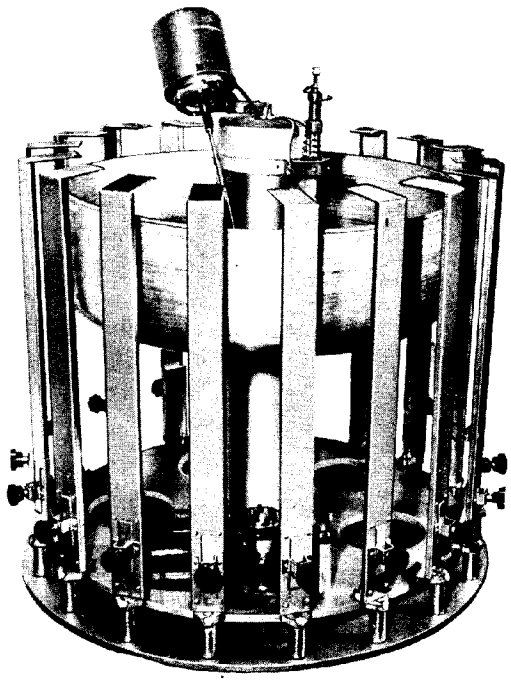

FIG. 18

Circular bath, shaking mechanism and manometer supports for microrespirometers.

The circular apparatus is to be recommended for its ease and speed of operation and its economy of space. It has been tested for several years and has proved remarkably free of mechanical troubles.

H. A. Lardy and R. H. Burris

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Chapter VI

DESIGN AND CALIBRATION OF FLASKS

It is of obvious importance to know the exact volume of flasks used on a microrespirometer. Several methods of calibration are described below together with methods of calculating and using flask constants.

CALIBRATION WITH MERCURY

Perhaps the simplest method of calibration is that of Krebs (1929). This method determines the flask volume to within 5% and is especially convenient for calibration of complicated flasks, e.g., Dixon-Keilin flasks. However, in practice this method is less accurate than the other methods described.

Although calibration with mercury may appear more laborious than other methods, it is the most accurate method available and is not particularly time consuming after one has had a little practice.

With a diamond point scratch a permanent reference mark about 1 cm. above the flask-to-manometer ground joint. Weigh the Warburg flask empty. Fill the flask with clean mercury, and by using a capillary pipette with a bent tip tease any small air bubbles trapped at the sides and bottom of the flask to the surface. Place the flask on the dry manometer joint and seat the joint. If there is too much mercury or insufficient mercury to rise to the mark scratched on the capillary manometer, remove or add mercury with a capillary pipette until the mercury just reaches the mark when the joint is seated. Immediately plunge a thermometer into the flask of mercury and record the temperature. Weigh the flask and mercury.

Bore a hole in a rubber stopper to fit over the manometer ground joint, and put the stopper about half way onto the joint; this forms a well at the inlet to the manometer. Pour mercury quickly into this well with the manometer in an inverted position. This procedure minimizes the possibility of getting air bubbles in the mercury column. Tip the manometer to allow mercury to flow into the graduated arm of the manometer. Open the stopcock and allow the mercury to drop into contact with the stopcock. Remove the rubber stopper thus dumping the excess pool of mercury (it is convenient to work over a shallow box or tray lined with a sheet of paper to catch mercury). Tip the manometer until the mercury column coincides with the scratch mark and record the reading on the graduated manometer column. Weigh the mercury in a tared weighing bottle. Repeat the above procedure, this time introducing a shorter continuous column of mercury. Tip the entire column into the graduated manometer tube and record the mm. of tubing that it occupies. Weigh this mercury. Accept room temperature as a reasonably accurate measure of the temperature of the mercury.

Calculate the volume of the Warburg flask to the scratch mark by dividing the weight of mercury by its density at the recorded temperature. In the same manner find the volume of the manometer from the scratch mark to the recorded level, and the volume of the measured length of manometer tubing. Determine the volume of the manometer tubing per mm. length, and with this value correct the volume measured from the scratch mark to give the volume from the scratch mark to the 250 mm. mark. Calibration at the 250 mm. point is most convenient for studies of gas uptake. From the volume determined per mm. tubing length the calibration can be shifted to any other reference point that proves useful; the 50 mm. point is suitable when gas output is to be followed.

The sum of the flask volume and the manometer volume to the 250 mm. mark minus the liquid volume to be used in the flask is the V_g to be substituted in the equation to determine the flask constant. For density of mercury consult Table XV, page 60.

Schale's (1944) procedure has been modified by Santiago Grisolia to permit separate calibration of flasks and manometers, since it is useful to have flasks calibrated on more than one manometer in case of breakage. Grisolia's method is as follows:

(a) Scratch a mark on the sidearm of the manometer above the ground glass joint.
(b) Loosely clamp the manometer to a ringstand at an angle of about 30° with the sidearm uppermost.
(c) Fill a short rubber tube fitted with a screw clamp with mercury and attach to the gas inlet tube of the manometer. The stopcock should be open and the rubber tube pushed on far enough so that the mercury almost comes to the sidearm scratch and to the reference point (usually 150 mm.).
(d) Tilt the manometer and adjust the screw clamp so that the mercury level is exactly at both the scratch mark and the reference point.
(e) Close the stopcock and pour out the mercury from the manometer into a tared receptacle. Weigh and calculate the gas volume of the manometer.
(f) Place the manometer upright, fill the flask to be calibrated with mercury, and insert its sidearm plug. Remove any air bubbles with the aid of a wire.
(g) Seat the flask on the ground joint of the manometer. If the mercury level is above or below the scratch mark on the manometer sidearm, remove or add small portions of mercury with the aid of a medicine dropper. Adjust until the level coincides with the mark when the flask is firmly seated.
(h) Pour the mercury into a tared vessel, weigh, and calculate the volume of the flask.
(i) Add the values from (e) and (h) to obtain the total volume ($V_g + V_f$) for a given flask and manometer.

This method has proved to be very efficient. After the manometer has been calibrated, further flasks may be calibrated with the manometer mounted and containing fluid.

R. H. Burris and R. W. McGilvery

CALIBRATION WITH WATER AND MERCURY

The manometer is emptied, and cleaned; with the stopcock closed, mercury is run into the ground joint while the manometer is inverted. By tilting the manometer it is easy to adjust the mercury level so that it is exactly at the 250 mm. point (or other reference point). The level of the mercury in the ground joint arm is then marked; this can be done conveniently with a thin strip of gummed paper. The mercury is emptied and weighed. This weight permits the calculation of the volume of the manometer from the 250 mm. point to the mark on the ground joint arm.

The clean dry flask with sidearm plugs in place is weighed. It is then filled with distilled water which has been boiled to remove dissolved gases and cooled. Water is added or removed until the level just reaches the mark on the manometer arm when the flask is firmly seated. It is well to add just enough water so that the level rises about a mm. above the mark--the excess can be removed by lowering the flask slightly and reseating; this will force a small quantity of the water between the glass joint and the flask whence it can be removed from the outside by absorption with a strip of filter paper. When the water has been adjusted to the mark, the flask is removed and weighed. From the temperature of the water its density can be ascertained, and the volume of the flask to the mark can be calculated. This added to the volume from the mark to the 250 mm. point gives the total volume of the system.

CALIBRATION FOR INTERCHANGEABILITY OF FLASKS

Allen (1948) has described a method whereby any flask can be used on any manometer without direct calibration for that manometer. This is particularly convenient when replacement is necessary. A flask is weighed and then filled with enough mercury to rise about 1 cm. into the manometer arm. It is then placed on the dry joint of the first manometer of a series and seated. A line is scratched with a diamond point at the top of the mercury column. One then repeats the procedure and marks the point to which the mercury rises on all other manometers of the series; care is taken to be sure that the temperature of the mercury does not change or that none is spilled. The manometer volumes are then calibrated to the mark as described under calibration with mercury. From this point on it is only necessary to calibrate any flask to the reference mark on any manometer, and

the V_g for that flask in combination with any of the other manometers of the series can be calculated readily.

W. W. Umbreit

CALIBRATION OF THE BARCROFT DIFFERENTIAL RESPIROMETER

The constant of the Barcroft differential manometer may be arrived at by three methods: (1) by calculation, using the simplified equation (equation 37, page 67); (2) by the Münzer and Neumann method and (3) by liberating or absorbing a known amount of gas in the reaction vessel by means of a chemical reaction. The first two methods are preferred by most workers, although the third has certain advantages. It cannot be too strongly emphasized that, whichever method is employed, the conditions under which the constant is determined must be the same as those which will prevail during the course of experimentation. If not, a correction must be applied.

Determining the constant by calculation: The usual case is to calculate the constant for O_2, i.e., K_{O_2}(NTP). 'A' is determined by running in sufficient mercury to form a 100-150 mm. column in the graduated portion of one side of the manometer. While holding the manometer by its ends to avoid a change in temperature, measure the length of the mercury column in 3-4 positions. Run the mercury into the other side of the manometer and repeat the measurements. The length of the column in the several positions serves as a measure of the uniformity of the bore of the capillary tubing. The volume of the mercury column is calculated from the weight and density of the mercury at the temperature of the room. 'A' then equals the volume in µl. divided by the average length in mm. The A values for both sides of the manometer should agree to within 5%; if not, it is desirable to check the K_{O_2} obtained by this method with one of the other two methods; method (2) is readily adaptable to determining the K_{O_2} value for different values of h, which covers this variation in cross-sectional area of the manometer tubes.

To determine the volumes of the vessels and their manometer tubes: An index mark is made on each manometer limb about 1 cm. above the ground-glass vessel-joint. Attach a small funnel to the stopcock capillary with a 25-40 cm. length of rubber tubing. While manipulating the manometer in an inverted position, add mercury through the stopcock capillary until one meniscus coincides with the index mark and the other meniscus coincides with the "zero" on the graduated portion of the manometer (the 15 cm. graduation). Of course, there must not be any trapped bubbles of air. The stopcock is now closed and the mercury in the manometer limb shaken out and weighed. The volume of the mercury is determined from its weight and density, and it represents the volume of the manometer limb from the "zero" of the manometer to the "index mark". The same procedure is followed to determine the volume of the other manometer limb. The volumes of the flasks, which have been labeled R and L respectively, are determined at the temperature of the bath. Using a bit of filter paper held with curved forceps, or a pipe cleaner, to remove bubbles of trapped air, fill each flask with sufficient mercury so that the mercury is forced up to the "index mark" on the manometer limb when the flask is attached to its manometer limb. Ordinarily this requires time and patience. The mercury-filled vessel is placed in a shallow dish (a tea-glass coaster works very well) and the whole supported in the bath with the neck of the vessel projecting above the water. Allow time for temperature equilibration. Take hold of the coaster and neck of the flask, and carefully work the neck of the flask onto the ungreased joint of its capillary limb. When this operation is properly carried out no mercury is trapped in the ground-glass joint. Usually the meniscus of the mercury does not coincide with the "index mark" on the first trial. Return the coaster-supported flask to the bath, and add or remove mercury from the flask with a capillary-tipped eye-dropper. After allowing time for temperature equilibration, again attach the flask to its manometer limb for another check on the coincidence of the mercury meniscus and "index mark". This is obtained by repeating the above procedure. The mercury is then weighed and its volume determined at the temperature of the bath. The main sources of error in this determination are: Air is often trapped below the ground-glass joint. It can be removed by rocking the top of the respirometer from side to side. The temperature of the glass and mercury may change considerably while the vessel is being attached to the manometer unless this operation is carried out quickly. Avoid contact between flask and hands as much as possible. Finally, check the final coincidence of the mercury meniscus

and "index mark" by submerging the end of the manometer limb with the attached flask filled with mercury in the bath.

By adding the volume of the vessel to the corresponding manometer limb volume, the total gas volume of one side of the respirometer is obtained. Determine the total gas volume of the other side in the same manner. These two gas volumes should be equal to within 0.1%. In most instances they will be unequal in volume. While it may help to switch the flasks, the quicker procedure is to pair flask and limb of manometer so that the volume of the reaction-vessel side of the respirometer is the smaller. Glass beads can then be added to the compensation-vessel side to obtain the same gas volume. The requisite volume of glass beads can be measured out by adding beads to water in a partially filled burette until the necessary volume is obtained. This "pairing" is unnecessary if it makes no difference which limb of the manometer carries the reaction vessel.

The gas volume of each side can now be obtained by subtracting the volume of the liquid (including that in the sidearms and center well, and the volume of the tissue) which will be used in the flasks from the total gas volume.

The density of the manometric liquid, e.g., iso-caproic acid, is determined at the temperature of the room by means of a pycnometer. P_o can then be defined in terms of mm. of manometric liquid.

The absorption coefficient, $\underline{\alpha}$, of oxygen in the liquid must be obtained from a table of "$\underline{\alpha}$ values" (see Chapter I), or determined by actual experiment. For most purposes the $\underline{\alpha}$ for oxygen in water is sufficiently accurate. '$\underline{\alpha}$' had best be determined or obtained from the literature for liquids other than water if the volume of liquid in the flask is more than 10 ml. It is possible to calculate the $\underline{\alpha}$ of oxygen, carbon dioxide, and nitrogen from the data of Geffken (1904) for salt solutions.

Having obtained the above data, the K_{O_2} of the respirometer may be calculated with the use of equation (37). The example below may be of help.

Details of Calibration: (a) General considerations: Room and bath temperatures, 25°C; iso-caproic acid as manometer liquid; flasks of Warburg type with two sidearms; volume of liquid in each flask, 4.2 ml. (3.0 ml. of nutrient solution, 0.5 ml. 5% glucose in each sidearm. 0.2 ml. 20% KOH in center well). The reaction vessel is to contain 100 µl. (0.1 ml.) of algal cells suspended in 3,000 µl. of nutrient solution.

(b) Determination of A.

Average length of H_g column in left side of manometer = 105.8 mm.
Average length of H_g column in right side of manometer = 107.6 mm.
Weight of mercury = 606.4 mg.

$$A = \frac{606.4}{106.7 \times 13.53} = 0.42 \text{ mm.}^2$$

(c) Determination of V_g and V'_g:

Wt. of H_g filling right flask and manometer limb = 251.3 gms.
Wt. of H_g filling left flask and manometer limb = 261.6 gms.

$$\text{Then } V_g = \frac{251.3 \times 1000}{13.53} - 4{,}200 = 14{,}372 \text{ µl.}$$

$$\text{And } V'_g = \frac{261.6 \times 1000}{13.53} - 4{,}200 = 15{,}134 \text{ µl.}$$

To reduce V'_g to the same volume as V_g, 760 µl. of glass beads are added to the left (compensation) vessel.

(d) Determination of P_o:

Wt. of 5.00 ml. of iso-caproic acid in a previously calibrated pycnometer = 4.605 gms.
Density of the acid = 0.921

$$\text{Then } P_o = \frac{760 \times 13.53}{0.921} = 11,164 \text{ mm.}$$

(e) The absorption coefficient, α, of $O_2 = 0.030$.

(f) Calculation of K_{O_2}:

$$K_{O_2} = h\left[\left(1 + \frac{0.42 \times 11,164}{2 \times 14,373}\right)\left(\frac{\frac{14,373 \times 273}{298} + 4,200 \times 0.030}{11,164} + \frac{0.42 \times 273}{2 \times 298}\right)\right]$$

and $K_{O_2} = h(1.61)$.

Determining the constant by the Münzer and Neumann Method: This method (Münzer and Neumann, 1917) requires a carefully calibrated 1 ml. pipette provided with a leveling bulb containing mercury, a calibration manometer of 3 mm. glass tubing filled with kerosene or light paraffin oil colored with a dye (Sudan IV is satisfactory), and a capillary glass manifold connecting the reaction-vessel side of the respirometer with the pipette and calibration manometer and bearing a stopcock at its free end. The set-up is illustrated diagrammatically in Fig. 19.

As arranged for a calibration, the set-up is much more compact than indicated by this diagram. The pipette with its leveling bulb, the manometer, and the manifold can be attached by means of spring clips to an inverted L-shaped metal rod which in turn is attached to the respirometer support. Such an arrangement permits the apparatus to be placed on the shaker with the respirometer vessels, the pipette, and the lower portion of the calibration manometer immersed in a glass-fronted, constant temperature water bath. A suitable water bath may be improvised from a large bell-jar.

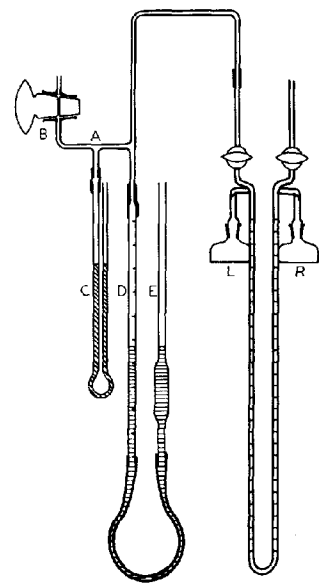

FIG. 19

Apparatus for Calibration by the Münzer-Neumann Method. A, manifold; B, manifold stopcock; C, calibration manometer; D, 1 ml. pipette; E, leveling bulb; R, reaction vessel; L, compensation vessel.

After having thoroughly cleaned and dried the vessels and manometer capillary of the respirometer (see Chapter 5), pipette into the respirometer capillary sufficient manometer liquid to fill the manometer limbs to, or approximately to, their 15 cm. marks when the manometer is in a vertical position. Attach the holder bearing the manifold, etc., to the respirometer support. Add sufficient mercury to the leveling bulb to allow for the displacement of the entire gas volume of the pipette. Add sufficient colored paraffin oil to the manometer to bring the level of the oil about 10 cm. below the opening into the manifold. Pipette into the respirometer vessels the correct volume of the liquid or liquids (include that in the sidearms, but substitute water for the KOH in the center well) which is to be used in determining the gas exchange of cells or tissue. Clean and replace the grease in the stopcocks of the manometer and manifold. Attach the apparatus to the shaker with the vessels, etc., submerged in the constant temperature water bath. Do not attach the manifold to the respirometer, or close any of the stopcocks. Allow 10-15 minutes for temperature equilibrium. The manifold is now connected to the reaction-vessel

side of the respirometer. After closing the stopcock of the manifold, alternately raise and lower the mercury in the pipette to mix the air in the apparatus. Repeat this operation several times over a period of 10-15 minutes. It is not necessary to shake the apparatus while this equilibration is being carried out.

The manifold stopcock is now opened, and the manometer and calibration manometer are checked for 'zero' reading. The following data are recorded: P (in mm. Hg at T°C.), room temperature, bath temperature, pipette reading and the height of the liquid in the right and left sides of the respirometer capillary. The manifold stopcock and the stopcock of the compensation-vessel side of the respirometer are now closed. Some 60-100 µl. of gas is withdrawn from the respirometer by lowering the mercury in the pipette. The stopcock of the reaction-vessel side of the respirometer is now closed, and the calibration manometer is again brought to 'zero' by raising the mercury in the pipette. At this point, it is best to allow the apparatus to stand for a few minutes to allow for restoration of equilibrium between the gases in the gas spaces and liquids in the vessels of the respirometer. After checking the 'zero' of the calibration manometer, record the height of the liquid in the right and left sides of the respirometer manometer and the pipette reading. The stopcock of the reaction-vessel side of the respirometer is now opened, and the mercury in the pipette adjusted to its original level. The respirometer and calibration manometer should be restored to their 'zero' levels. If they are not, a leak, a change in temperature, or a change in barometric pressure during the calibration is indicated.

The above procedure is carried out several times, and the ratio of the volume of gas removed (or added) to h is calculated. Usually these agree to within 1%. The average ratio obtained is then substituted in the following equation in order to correct the value of the constant obtained under the conditions of calibration to 0°C. and 760 mm., where

$$K_c = \left(\frac{\text{Vol. of gas}}{h} \right) \left(\frac{273}{T_c} \right) \left(\frac{P_c - p_c}{P_o} \right) \qquad (27)$$

the subscript c refers to the value obtaining under conditions of calibration.

This equation expresses the relation between the µl. of gas (NTP) removed, or added, and the reading of the respirometer.

It might be questioned if this type of calibration, with the vessels containing water and air, holds for experimental liquids and gas mixtures. By choosing an α value for oxygen in water, it can be shown that the value of $V_f \alpha$ in those cases where V_f is of the order of 3000-5000 µl. is practically negligible in calculating k_{O_2} by use of the simplified equation. The α O_2 for experimental liquids will not be greater, hence their presence in the vessels will not alter the value of K_c. Then the results of the calibration can be applied directly to the determination of oxygen. The above statements, however, do not apply to the very soluble CO_2 gas. In this case the calibration had best be made using the simplified equation.

As described above in the presentation of the theory, K_c does not vary with the temperature. On the other hand, if the $V_f \alpha$ term in the simplified equation is omitted the constant becomes exactly proportional to $1/T$. Then, to use the respirometer at an experimental temperature, $T_{exp.}$, which differs from T_o, a correction must be applied. Thus,

$$K_{exp.} = K_c \frac{T_c}{T_{exp.}}$$

Under experimental conditions, changes in barometric pressure and in the vapor pressure of water usually occur. Then, since K_c is inversely proportional to gas pressure,

$$K_{exp.} = K_c \left[\frac{T_c}{T_{exp.}} \; \frac{P_c - p_c}{P_{exp.} - p_{exp.}} \right]$$

and correcting $K_{exp.}$ to give µl. of dry gas at NTP,

$$K_{exp.} = K_c \left[\left(\frac{T_c}{T_{exp.}} \right) \left(\frac{273}{T_c} \right) \left(\frac{P_c - p_c}{P_{exp.} - p_{exp.}} \right) \left(\frac{P_{exp.} - p_{exp.}}{P_o} \right) \right]$$

or

$$K_{exp.} = K_c \left[\frac{273}{T_{exp.}} \right] \left[\frac{P_c - p_c}{P_o} \right]$$

and

$$x_{O_2} = h \left[K_c \left(\frac{273}{T_{exp.}} \right) \left(\frac{P_c - p_c}{P_o} \right) \right] \qquad (28)$$

which is the complete equation to be used with the Münzer and Neumann method of calibration.

Details of a calibration (and a test of the constancy of K_c):

(a) Determination of K_c: room temperature, 25°C.; manometer liquid, isocaproic acid; large size (ca. 60 ml.) Barcroft type flasks; 36 ml. water in each flask; P, 739.0 mm. Hg, 25°C., brass scale barometer.

T °C.	h_c mm.	x_c µl.	K_c
10	28.8	81	2.72
15	45.2	122	2.70
20	68.3	186	2.71
25	34.8	95	2.73

These data are averages of 3-4 determinations made at each temperature.

(b) Calculation of x_{O_2}: room temperature, 25°C.; bath temperature, 25°C.; P, 735.5 mm. Hg (corrected).

$$K_{O_2} = 2.73 \left(\frac{273}{298} \right) \left(\frac{735 - 23.7}{760} \right)$$

$$K_{O_2} = 2.34$$

$$x_{O_2} = h(2.34)$$

Calibration of the Warburg respirometer by the Münzer and Neumann method: The apparatus, etc., required and the procedure are essentially the same as those used in calibrating the Barcroft differential respirometer.

In calibrating the Warburg respirometer by this method, the pipette, manometer and manifold are mounted on the vessel-side of the respirometer support. The manifold is attached to the vertical capillary on the vessel-side of the respirometer which is equipped with its empty vessel and the requisite amount of Brodie manometer fluid. After allowing sufficient time for the gas (air) in the vessel and pipette to reach temperature equilibrium in the constant temperature bath, both manometers are leveled (the respirometer manometer at 250 mm.), the two stopcocks are closed (the three-way stopcock of the respirometer is turned so that the gas space of the respirometer communicates with that of the manifold), and some 50-100 µl. of gas are withdrawn from the respirometer by lowering the mercury in the pipette. The respirometer stopcock is now turned to disconnect the gas space of the respirometer from that of the manifold but not to connect either gas space to the atmosphere. After returning the Brodie's solution to its former level in the

closed side of the manometer, the difference in height of the fluid in the open side of the manometer, i.e., h, is recorded. The manifold manometer is now leveled, and the amount of gas removed from the respirometer as measured by the pipette recorded. The temperature of the bath and the barometric pressure of the atmosphere are also recorded.

The above procedure is carried out several times until the operator is assured by the constancy of the ratio of the volume of gas removed to h, which should agree to within 1%, of the accuracy of the determinations of these two sets of values.

The total gas volume, V, of the Warburg respirometer at the temperature of calibration may now be calculated by substituting any of the acceptable corresponding values of "volume-of-gas-removed" and h in the following equation:

$$V = \frac{\text{Vol. gas removed} \times P}{h} \qquad (29)$$

where P is atmospheric pressure (corrected) in mm. of manometer fluid.

The K_{O_2} and K_{CO_2} of the respirometer may now be obtained through the use of the equation given below under the heading of "Flask Constants".

If desired, the K_{O_2} of Warburg respirometers may be obtained directly with the Münzer and Neumann method. In this case the vessel of the respirometer contains the same volume of the liquid which will be used in experiments, including any which will be used in the sidearm, but water is substituted for the KOH in the center well. Proceed to determine several sets of values of "volume-of-gas-removed" and the corresponding h's. The average value of the ratio of the two, where individual values agree to within 1%, is substituted in equation (27) to obtain K_{O_2}. This constant of course applies only when the experimental temperature is the same as the temperature of calibration. It may be corrected for use at other experimental temperatures by means of equation (28).

J. F. Stauffer

FLASK CONSTANTS

As derived in Chapter 1, the relation between the difference in reading observed on the Warburg manometer (h) and the gas change within the system (x) is:

$$x = h\left[\frac{V_g \frac{273}{T} + V_f\alpha}{P_o}\right] = hk \text{ where } k = \left[\frac{V_g \frac{273}{T} + V_f\alpha}{P_o}\right]$$

Since for any given experimental condition, the gas volume of the flask (V_g) the fluid volume of the flask (V_f), the temperature (T), the solubility of the gas in the fluid (α), and the pressure (in mm. of manometer fluid) of 1 atmosphere (P_o) are all constant, the value "k" (the "flask constant") is constant for any given set of experimental conditions and varies only as the experimental conditions vary.

FACTORS FOR DIFFERENT VOLUMES OF FLUID IN THE WARBURG FLASKS

One can, of course, calculate the constant to be employed for any set of experimental conditions from the equation above. However, there is a simple relationship between the constant at one set of conditions and the constant at the same conditions except for the presence of one more ml. fluid in the flask. We will define this change in constant as Δ ml.

$$\Delta \text{ml.} = k' - k = \frac{(V_g - 1000)\frac{273}{T} + \alpha\,(V_f + 1000)}{P_o} - \frac{V_g \frac{273}{T} + \alpha\, V_f}{P_o}$$

(k' = flask with 1 ml. more fluid in it)
(k = original flask)

$$\Delta \text{ml.} = \frac{-1000\frac{273}{T} + 1000\,\alpha}{P_o}$$

Thus, Δml. is independent of V_g or V_f (i.e., factors governed by the size of the flask or the amount of fluid in it) and dependent only upon α and T. For any temperature the Δml. is calculated easily.

Temperature °C	Δml. O_2*	Δml. CO_2*
20	-0.090	-0.005
28	-0.088	-0.020
33	-0.087	-0.026
37	-0.086	-0.031
43	-0.084	-0.037

*For each additional ml. of fluid in the flask the factor changes by this amount.

For example, a flask has a k_{O_2} (37°C.) of 1.69 when it contains 3 ml. of fluid. If this flask were to contain 4 ml. of fluid its factor would be: $k_{O_2} = 1.69 - 0.086 = 1.60$. If it were to be run with 1.5 ml. of fluid its factor would be: $k_{O_2} = 1.69 + (1.5 \times 0.086) = 1.69 + 0.13 = 1.82$.

CALCULATIONS OF FACTORS FROM K_e

Returning to the original equation for calculating the flask constant:

$$k = \frac{V_g \frac{273}{T} + \alpha\, V_f}{P_o}$$

it will be noted that if there is no fluid in the flask its constant (here labeled k_e) is:

$$k_e = \frac{V_g \frac{273}{T}}{P_o}$$

This constant is independent of the gas involved and depends only on the volume of the flask and the temperature. Further, the term $\frac{273}{T}$ is a constant (c) dependent only on the P_o

temperature. This constant (for Brodie's solution in the manometer, i.e., $P_o = 10{,}000$) has been recorded in the following table:

Temperature °C.	c
20	0.0932
25	0.0916
28	0.0907
30	0.0901
37	0.0881

Therefore, one may calculate readily any flask constant for any condition as follows:

$$k = k_e + (\Delta \text{ml.})\ (\text{ml. fluid})$$

$$= (V_g)\ (c) + (\Delta \text{ml.})\ (\text{ml. fluid})$$

Thus at 37°C., flask volume 13.5 ml.:

$$k_e = 13.5 \times 0.0881 = 1.19$$

$$k_{O_2}\ [3 \text{ ml. fluid}] = 1.19 + (-0.086 \times 3) = 1.19 - 0.26 = 0.93$$

$$k_{O_2}\ [2 \text{ ml. fluid}] = 1.19 + (-0.086 \times 2) = 1.19 - 0.17 = 1.02$$

$$k_{CO_2}\ [3 \text{ ml. fluid}] = 1.19 + (-0.031 \times 3) = 1.19 - 0.09 = 1.10$$

The MacLeod and Summerson (1940) graphical method of determining flask constants is based on the same principles as govern the calculations from k_e and Δml. If the k_e (0 ml.) and the k for any other volume (e.g., 3 ml.) are calculated and these constants are connected by a straight line on a graph with ml. fluid as the abscissa and flask constant as ordinate, the constant for any liquid volume can be taken from the line. The lines for a given temperature have the same slope, but a different intercept for each flask. With a series of lines for a given flask, at 10° temperature intervals, flask constants at any liquid volume and temperature can be obtained by inspection.

It should be noted also that the difference between the k_{O_2} and k_{CO_2} (or the constants for any other gases) is a constant under the same experimental conditions. In the equation:

$$k = \frac{V_g \frac{273}{T} + \alpha\ V_f}{P_o}$$

the nature of the gas influences only the factor α. Thus, at 37°C. and with 3 ml. of fluid in the flask the term $\frac{\alpha V_f}{10{,}000}$ for O_2 is:

$$\frac{0.024 \times 3{,}000}{10{,}000} = 0.0072$$

and for CO_2 is:

$$\frac{0.57 \times 3{,}000}{10{,}000} = 0.171$$

and the difference is: $0.171 - 0.007 = 0.164$

The k_{CO_2} for any flask with 3 ml. of liquid at 37°C. is 0.164 higher than the k_{O_2} for the flask. If the k_{O_2} values are known for a set of flasks the k_{CO_2} values for the same conditions can be calculated merely by adding a constant.

W. W. Umbreit

NOMOGRAPHS FOR WARBURG FLASK CONSTANTS

Dixon (1945) has published nomographs for the calculation of flask constants for both the constant volume and differential respirometers. The use of either a nomograph or the rapid calculation from k_e and ml. greatly simplify calculations. The nomograph (Fig. 20), for the Warburg constant volume respirometer, given here covers the usual flask volumes encountered.

To use the nomograph (Fig. 20) to calculate the k_{O_2} for a flask, connect the volume of the fluid in ml. (scale A) with the temperature in °C. (scale B) and read the product where the straightedge intersects scale D. Then connect the gas volume in ml. (scale J) with temperature in °C. (scale I) and read the product at the point that the straightedge intersects scale H. Add these two products longhand or add them by connecting the products on scales D and H with a straightedge and reading the sum on scale G.

To calculate k_{CO_2} connect the volume of fluid in ml. (scale A) with the temperature (scale C) and read the product at the point the straightedge intersects scale E. Connect scales J and I and read on H as for determining k_{O_2}. Add the products longhand or connect the product on scale E with the product on scale H and read the sum on scale F.

R. H. Burris

CONSTANTS USED IN THE CALIBRATION OF FLASKS

TABLE XV

Temperature	Density* of Hg	Water	α O_2	α CO_2	α H_2	α CO	α N_2
20	13.5462	0.9982	0.0310	0.878	0.0182	0.0232	0.0154
25	13.5340	0.9971	0.0283	0.759	0.0175	0.0214	0.0143
30	13.5217	0.9957	0.0261	0.665	0.0170	0.0200	0.0134
35	13.5095	0.9941	0.0244	0.592	0.0167	0.0188	0.0126
37	13.5046	0.9934	0.0239	0.567	0.0166	0.0184	0.0123
40	13.4973	0.9922	0.0231	0.530	0.0164	0.0177	0.0118

*Density = grams/ml.

TABLE XVI

Temp.	c*	Δml**	
		O_2***	CO_2
20	0.0932	-0.090	-0.005
25	0.0916	-0.089	-0.016
30	0.0901	-0.087	-0.023
35	0.0886	-0.086	-0.029
37	0.0881	-0.086	-0.031
40	0.0872	-0.085	-0.034

*$k_e = V_g\ c$ see page 58. **see page 58.

***The same values may be used for N_2, H_2.

W. W. Umbreit

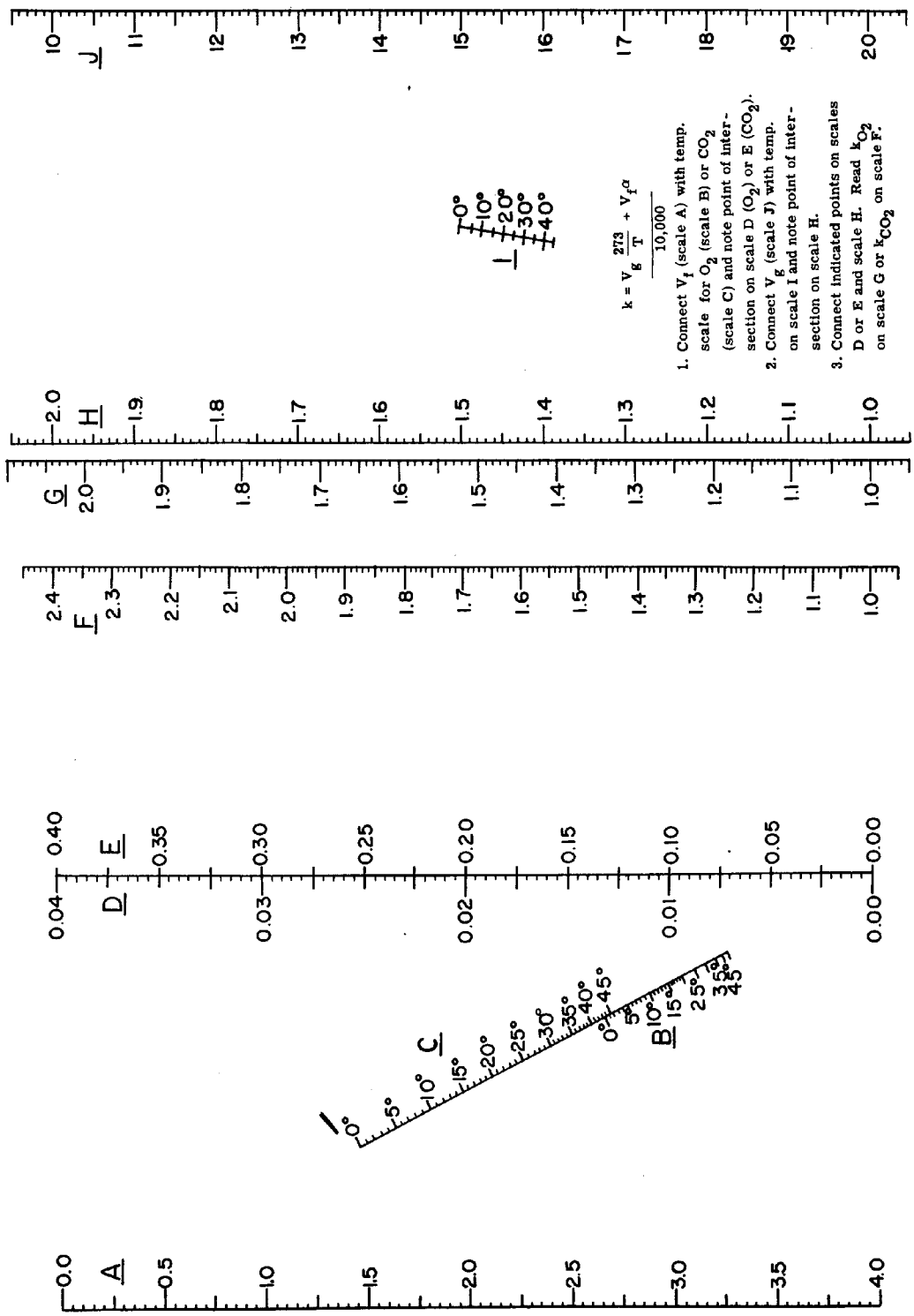

FIG. 20

Nomograph for the calculation of Warburg flask constants.

DESIGN OF WARBURG FLASKS

Two shapes of flasks are in common use. These are illustrated in Fig. 21. Flasks of type A (with angle edges) tip less easily when detached from the manometer and are suitable for use on manometric instruments which employ a reciprocating motion whereby the flask is moved to and fro in the same plane. When used on the type of instrument in which the pivot of the manometer is at the bottom and the flask is rocked back and forth, tissue tends to collect at the edge of the flask. Type B (with rounded edges) may be used equally well with either type of shaking device, but, because the bottom is not flat, it tends to tip more easily. Type A is also preferred for irradiation work.

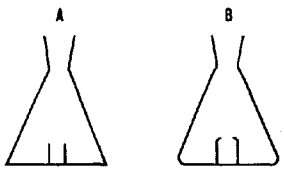

FIG. 21

Different shapes of Warburg flasks.

An additional modification has been suggested by Dr. Cohen. If the center well is slightly constricted at the top (as shown in "B", Fig. 21), splashing or "creeping" of alkali into the main compartment is largely prevented.

When there is a very limited supply of the enzyme under study, it is frequently desirable to employ unusually small reaction vessels. The most frequently used vessels have a volume of about 15 ml., but flasks of 7 to 8 ml. volume also are available commercially. A reaction in these vessels with half the usual amount of materials will cause a shift in the manometric fluid equivalent to that observed with the usual flasks and the usual amount of reactants; the precision of measurement in the two cases will be essentially the same.

A flask (Fig. 22) with a standard taper sidearm opening has been well adapted to some of our work with N_2^{15} (Burris, et al., 1943). The flasks have been used on a stationary manifold which does not allow tipping of the flasks. However, when sidearm B is inserted directly into the outer joint on the flask, its contents can be added directly to the main chamber of the flask by rotating the sidearm (rotating sidearms have been used for many years on Dixon-Keilin flasks). If it is necessary to mix materials in a sidearm, sidearm A is inserted into the flask and sidearm B is inserted into A; rotation of B adds material to A. A standard taper plug is inserted into the main flask sidearm outlet when no sidearm is required. For work under aseptic conditions a cotton plug is placed below the top ground joint and is retained by the indents at the base of the bulged section.

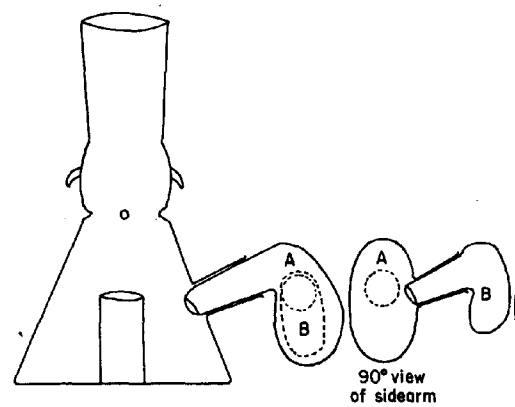

FIG. 22

Flask with rotating sidearms.

In the isolation of intermediates of a reaction it is often helpful to increase the amount of the reactants and to carry on the reaction and measurements in flasks of approximately 125 ml. volume. Flasks very similar to the small Siamese sidearm flasks (Fig. 30) are useful; they are modified so that (1) although the primary sac of the double sidearm is an integral part of the flask, the secondary sac can be rotated (as sac B, Fig. 22), and (2) the neck of the flask can be plugged with cotton (as in Fig. 22). It is impossible to rinse the substrate from the simple sidearm of the usual Siamese type flask without mixing the contents of the Siamese sidearm, but with the modified flask the rotating sac on the Siamese sidearm can be turned back and forth so that such rinsing of the simple sidearm can be accomplished without mixing in the Siamese sidearm. When a cotton plug is inserted the flask can be sterilized and kept aseptic during a run. The sidearm openings are plugged with

cotton during the sterilization; when a run is set up the cotton plugs are discarded and replaced by the sterile glass plugs which are lubricated with sterile grease. When the secondary sac of the Siamese sidearm is not required the opening is closed with a standard taper plug.

The use of aseptic technique may be dictated by the desire to keep contaminants from the system or to prevent the spread of pathogens. If standard flasks only are available, an adapter (suggested by J. B. Wilson) is useful for work with pathogens. This adapter (Fig. 23) consists of an outer joint to fit the manometer, an inner joint to fit the flask, and an intervening space carrying a cotton plug. The adapter, the sidearm plugs, and the cotton plugged flask are sterilized separately, the flask is filled using normal bacteriological precautions, the cotton plug from the flask is discarded, and the cotton plugged adapter lubricated with sterile grease is connected to the flask and manometer. The sterile sidearm plugs are lubricated with sterile grease before inserting them. After the run the flask and adapter are removed and sterilized (by autoclaving or preferably by immersion in a disinfectant) as a unit before opening.

R. H. Burris and W. W. Umbreit

FIG. 23

Adapter for preventing entry or escape of bacteria from reaction vessel.

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Chapter VII

THE DIFFERENTIAL MANOMETER WITH SPECIAL REFERENCE TO ITS USE IN STUDIES OF PHOTOSYNTHESIS

The differential respirometer was introduced by Barcroft (1908), and it is often called the "Barcroft respirometer", or "Barcroft manometer". The term respirometer is somewhat misleading however, since this type of apparatus has been used in a number of laboratories for measuring the rate of photosynthesis. It will suffice to point out that any type of apparatus designed to measure the uptake of a gas in terms of the change in the reading of a manometer can serve equally well to measure the evolution of the gas. Of course, in either case all conditions affecting the gas exchange must be taken into consideration.

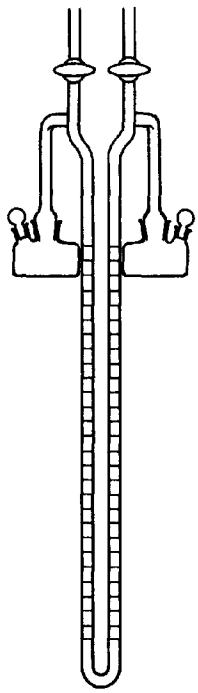


FIG. 24

The differential manometer.

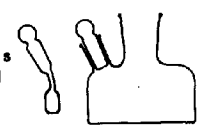
The differential respirometer is shown in Fig. 24. It is essentially a closed system formed of two flasks connected by a manometer. In practice the volumes of liquid and gas on both sides of the manometer are the same. One flask contains the cells or tissue and is called the reaction vessel (on the right, R). The other flask (on the left, L), free from cells or tissue, is called the compensation vessel, i.e., it serves to compensate for changes in temperature and barometric pressure during the course of an experiment. It is readily understood that a change in temperature will have the same effect (on the volume and pressure, and on the solubility of the gas being measured) in each flask and thus will produce no change in the height of the manometer liquid. Also, the use of the compensation vessel to form a closed system makes the manometer readings independent of any changes in barometric pressure which may occur during an experiment. Therefore, a thermobarometer is unnecessary.

CONSTRUCTION AND MOUNTING

The respirometer is constructed entirely of pyrex glass, preferably with standard interchangeable ground glass joints for the stopcocks and for the connection between each flask and limb of the manometer. The manometer, including the stopcock connections and the limbs carrying the flasks, is of thick-walled capillary tubing of uniform bore. The upright portion of the manometer is about 45 cm. in length. Each side is graduated at the same level in millimeters, with numbered centimeter graduations, over a 30 cm. portion. The two symmetrical halves of the respirometer are constructed as nearly alike as possible in respect to the internal diameter of the capillary tubes and gas volume. There is no restriction on the design of the flasks provided the gas exchange of the cells or tissue is not hindered. Many laboratories use the standard Warburg flasks with sidearms and center well. For photosynthesis measurements, flasks similar to that represented in Fig. 25 are used. Generally, the flasks do not have a volume of more than 40 ml.

FIG. 25

Flask for use in photosynthesis measurements. The paddle attached to the vent plug facilitates stirring (Emerson and Lewis, 1941).



The respirometer is mounted on an inverted L-shaped wood or metal support which in turn is attached to a suitable shaking device. As mounted, the graduated portions of the manometer tubes are vertical. The flasks are side-by-side and submerged at least 2 cm. beyond their necks in a constant temperature bath.

Three types of shaking devices are in use at present: (1) the older method of rocking the vessels from side to side; (2) the more recent method of short side-to-side swings

(Dixon, 1943); (3) a special type of shaking device whereby the flasks are rotated a few millimeters off-center (Warburg and Negelein, 1922). The second type of shaking device is common in all respiration laboratories; the third type is in use in photosynthesis laboratories, especially where the tissue or cell suspension must be irradiated, and the quantity of radiant energy absorbed determined. Regardless of the type, all of these shaking devices are constructed to allow considerable variation in rate of shaking and in the amplitude of the swing, or rotation, of the flasks.

THEORY

The theory of the apparatus has been described and elaborated on a number of times since the original account by Barcroft (1908). The most complete description of the theory for use in measurements of photosynthesis and respiration is given by Warburg and Negelein (1922). Dixon (1943) has also published a very complete description together with several extensions and modifications. The following account is based on those of Dixon and Warburg and Negelein.

Let: R = Reaction vessel.
L = Compensation vessel.
V_g = µl. of gas in the reaction-vessel side of the manometer.
V'_g = µl. of gas in the compensation-vessel side of the manometer.
V_f = µl. of liquid in the reaction vessel (including the tissue).
V'_f = µl. of liquid in the compensation vessel.
A = Cross-sectional area of the manometer capillary, in mm^2.
h = Manometer reading in mm. (the difference in height of the manometer liquid in the limbs of the manometer).
P_o = Normal pressure (760 mm. Hg), in mm. manometer liquid.
P = Atmospheric pressure (corrected), in mm. of manometer liquid.
ΔP = Increase in pressure of the confined gas in the reaction vessel.
$\Delta P'$ = Increase in pressure of the confined gas in the compensation vessel.
p = Pressure of water vapor in the gas spaces, in mm. manometer liquid.
T_o = 273° Absolute.
T = Temperature of the gas and liquid in the vessels in absolute degrees.
$\underline{\alpha}$ = Solubility coefficient of the gas produced or absorbed (µl. per µl. of liquid).

Suppose the apparatus contains the same volumes of gas and water on both sides of the manometer, and that the manometer liquid is at the same height in the two limbs of the manometer. The amount of gas (at 0°C., 273° Abs., and 760 mm. Hg; or NTP) in each gas space is:

$$\text{For R} = V_g \frac{273}{T} \frac{P - p}{P_o} \qquad \text{For L} = V'_g \frac{273}{T} \frac{P - p}{P_o} \qquad (30, 30')$$

If x amount of a gas is evolved in R, the manometer reading will be h. The increase in volume of the gas space in R is then $\frac{h}{2}$ A; the decrease in volume of the gas space in L is also $\frac{h}{2}$ A. But h does not indicate the true value of ΔP of the gas in R because of the compensatory effect of the rise of pressure in L. In other words, both pressure and volume increase in R while the pressure increases and the volume decreases in L. Taking these changes into consideration, the final volumes of the gas spaces will be:

$$\text{for R} = (V_g + \frac{h}{2} A) \left(\frac{P + \Delta P - p}{P_o}\right) \qquad \text{for L} = (V'_g - \frac{h}{2} A) \left(\frac{P + \Delta P' - p}{P_o}\right) \qquad (31, 31')$$

Since the pressure has increased in each vessel, the increase in the amount of gas dissolved in the liquid must be taken into account. It is:

$$\text{for R} = V_f \underline{\alpha} \frac{\Delta P}{P_o} \qquad \text{for L} = V'_f \underline{\alpha} \frac{\Delta P'}{P_o} \qquad (32, 32')$$

Summarizing: The amount of gas, x, produced in R is equal to the sum of the final volume of the gas space and the increase in the amount of the gas dissolved in the water less the original volume of the gas space; or,

$$x = (V_g + \frac{h}{2} A)\left(\frac{273}{T}\frac{P + \Delta P - p}{P_o}\right) + \left(V_f \alpha \frac{\Delta P}{P_o}\right) - \left(V_g \frac{273}{T}\frac{P - p}{P_o}\right)$$

$$\text{and } x = V_g \frac{273}{T}\frac{\Delta P}{P_o} + \frac{h}{2} A \frac{273}{T}\frac{P + \Delta P - p}{P_o} + V_f \alpha \frac{\Delta P}{P_o}$$

$$\text{and } x = \Delta P \frac{V_g \frac{273}{T} + V_f \alpha}{P_o} + \frac{A}{2}\frac{273}{T}\frac{(P - p)\frac{h}{\Delta P} + h}{P_o} \qquad (33)$$

On the other hand, no gas was produced in L; but the changes in volume and pressure must be accounted for in arriving at a value for P in order to solve equation (33).

Thus,

$$0 = (V'g - \frac{h}{2} A)\left[\frac{273}{T}\frac{P + \Delta P' - p}{P_o}\right] + \left[V'f \alpha \frac{\Delta P'}{P_o}\right] - \left[V'g \frac{273}{T}\frac{P - p}{P_o}\right]$$

$$\text{or, } 0 = V'g \frac{273}{T}\frac{\Delta P'}{P_o} - \frac{h}{2} A \frac{273}{T}\frac{P + \Delta P' - p}{P_o} + V'f \alpha \frac{\Delta P'}{P_o}$$

$$\text{or, } 0 = \Delta P'\left[\frac{V'g \frac{273}{T} + V'f \alpha}{P_o} - \frac{A}{2}\frac{273}{T}\frac{(P - p)\frac{h}{\Delta P'} + h}{P_o}\right]$$

$$\text{and } \Delta P' = h\left[\frac{\frac{A}{2}\frac{273}{T}(P + \Delta P' - p)}{V'g \frac{273}{T} + V'f \alpha}\right] \qquad (34)$$

As the pressure was originally the same on both sides of the manometer and as the pressure has increased by ΔP and $\Delta P'$ in R and L respectively, the difference in pressure as measured on the manometer is,

$$h = \Delta P - \Delta P'$$

$$\text{Or,}^* \Delta P = h + \Delta P' \qquad (35)$$

Substituting $\Delta P'$ of equation (34) in equation (35),

$$P = h + h\left[\frac{\frac{A}{2}\frac{273}{T}(P + \Delta P' - p)}{V'g \frac{273}{T} + V'f \alpha}\right]$$

$$\text{or, } P = h\left[1 + \frac{\frac{A}{2}\frac{273}{T}(P + \Delta P' - p)}{V'g \frac{273}{T} + V'f \alpha}\right]$$

And substituting this value of ΔP in equation (33) results in the complete expression for the differential respirometer:

*This equation is for respirometers with vertical manometers. If the manometer is tilted, then $\Delta P = h \cos \theta + \Delta P'$ where θ is the angle of tilt.

$$x = h \left[1 + \frac{\frac{A}{2}\frac{273}{T}(P + \Delta P' - p)}{V'_g \frac{273}{T} + V'_f \alpha} \right] \left[\frac{V_g \frac{273}{T} + V_f \alpha}{P_o} + \frac{A}{2}\frac{273}{T}\frac{(P - p)\frac{h}{\Delta P} + h}{P_o} \right] \qquad (36)$$

For the usual type of differential respirometer V_g (and V'_g) is about 30,000 µl., V_f (and V'_f) is about 3,000 µl., and A is always less than 0.5 mm^2; ΔP (and $\Delta P'$) will probably never differ from h by more than 50%; the maximum value of h is 300 mm; P will rarely be less than 95% of P_o; T will rarely be greater than 37.5°C.; p will be less than 5% of P; also, $V_f \alpha$ (and $V'_f \alpha$) is not more than 5% of the value of V_g (and V'_g), even for the very soluble CO_2 gas.

If it is assumed that these various quantities have their maximum values as stated above, by substituting in equation (36) it is found that,

$$x = h\ (1 + 0.07)\ (2.9 + 0.13)$$

This shows that the second term is about 1/14 of the first and the fourth term is about 1/20 of the third. Such a trial calculation proves that it is permissible to substitute P for $(P + \Delta P' - p)$, to omit $V'_f \alpha$ in the second term, and to omit the last half of the fourth term of equation (36) without introducing an error of more than 1%. Thus, we obtain the simplified equation,

$$x = h \left[\left(1 + \frac{A\ P_o}{2\ V'_g}\right) \left(\frac{V_g \frac{273}{T} + V_f \alpha}{P_o} + \frac{A}{2}\frac{273}{T}\right) \right] \qquad (37)$$

where the product of the terms within the brackets is the "constant" of the respirometer. To obtain the volume, x, of the gas evolved at NTP it is necessary only to multiply the value of the constant by the manometer reading, h.

Dixon (1943) has pointed out that a description of the theory, such as that given above, which assumes that the whole apparatus is at the temperature T actually reduces the error of the constant as determined by equation (37); furthermore, the use of p at T instead of at room temperature (as should be the case) introduces an error of less than 0.5% in the constant. It was also assumed that the gas spaces were filled with the same kind of gas as that evolved. It might be supposed that the pressure of a second gas would affect the constant due to the fact that with the increase in volume, $\frac{h}{2}A$, the partial pressure of the second gas would be reduced and some of the gas would pass from the liquid into the gas space. However, the reduction in partial pressure of a contained gas due to the increase in volume is very small. Even for a relatively high concentration of a very soluble gas, i.e., 10% CO_2, the error introduced is less than 0.1%; the presence of 80% N_2 has even less effect.

Details and examples of calibration of the Barcroft differential respirometer by calculation, using the simplified equation (equation 37), and by adding or removing known volumes of gas (Münzer and Neumann method) are presented in Chapter 6 (pages 52 to 56).

APPLICATIONS OF THE DIFFERENTIAL RESPIROMETER

RESPIRATION

The differential respirometer may be used in the same manner as the Warburg respirometer in determining oxygen uptake by the "direct method", i.e., alkali in the center wells, with or without liquid in the sidearms, of the flasks; K_{O_2} is obtained by calculation using the simplified equation, or the Münzer and Neumann method.

The differential respirometer may also be used to determine respiration by the "indirect method" of Warburg (see Chapter 4). Two respirometers are required. Unequal vol-

umes of liquid and unequal volumes of gas, unequal volumes of liquid and equal volumes of gas, or equal volumes of liquid unequal volumes of gas may be used in the reaction vessels of the two respirometers. K_{O_2} and K_{CO_2} may be obtained as described above, and x_{O_2} and x_{CO_2} obtained by use of the equations developed for use with the indirect method.

In general, the differential respirometer can be used in any situation to which the Warburg respirometer is applicable; it can be used in those cases where it is desirable to increase the pressure of the gas in the reaction vessel above the pressure of the atmosphere.

The differential manometer has frequently been used in the past in attempts to measure the "differential effect" of some treatment. For example, tissue would be added to both flasks, but glucose to only one. The use of the differential manometer in this manner is not desirable. Frequently it robs the investigator of just that data which may be of vital importance, i.e., what does the tissue do without treatment?

FERMENTATION

The differential respirometer is used without alkali in the center wells (if the flasks possess these) and with or without liquids in the sidearms of the flasks. K_{CO_2} is obtained by using the simplified equation (i.e., Number 37).

PHOTOSYNTHESIS

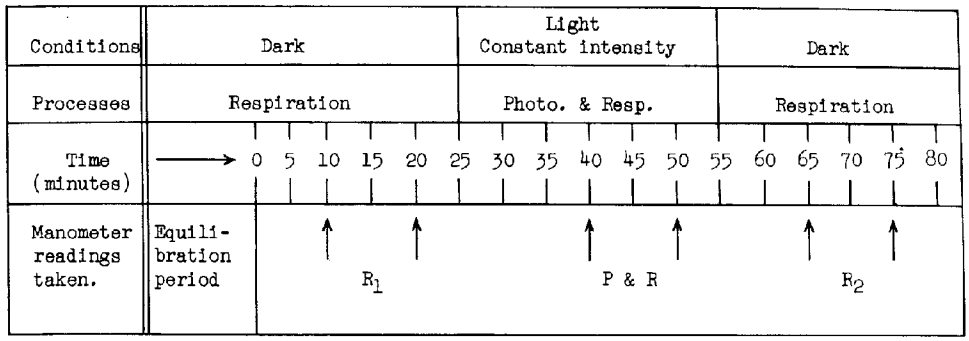
The discussion in this section is based on a consideration of photosynthesis as: carbon dioxide uptake and oxygen production by green cells in light.

In measuring photosynthesis the experiment usually involves three separate determinations of gas exchange; (1) a determination of respiration during a dark period, (2) a determination of gas exchange during the period the cells are illuminated, (3) a determination of respiration following the light period. These determinations are carried out in the order given. It is readily understood that the gas exchange between the cell and its environment during the illumination period results from both photosynthesis and respiration, i.e., the observed rate of photosynthesis is less than the true rate because of the evolution of carbon dioxide and the uptake of oxygen by respiration which continues during the time the cells are illuminated. There is no way of determining respiration alone for illuminated green cells under experimental conditions where carbon dioxide is present, and it is known that the metabolism of Chlorella cells is not the same in the absence and in the presence of carbon dioxide (Emerson, Stauffer and Umbreit, 1944). Hence, respiration is measured immediately before and after the illumination period and the average value of these two determinations added to the observed rate of photosynthesis to give the true rate of photosynthesis.

As a matter of fact, in experiments seeking to establish the relationship between light absorbed and oxygen produced (CO_2 assimilated) the suspension of green cells is oftentimes so dense that, even though all of the light is absorbed, what one actually measures is a decrease in the rate of respiration during the time the cells are illuminated (c.f., Warburg and Negelein, 1922).

The following discussion will serve to distinguish two variations of the above described method of obtaining the true rate of photosynthesis. Both have proved satisfactory in determining the quantum efficiency of photosynthesis using the alga Chlorella.

A. "Steady state of gas exchange" method: The following diagram indicates the general relationships:



The duration of the light and dark periods may be varied, although 30-60 minute periods appear to be satisfactory. The R_1 and R_2 periods, which may differ in duration, are the same as or some proportion of the P & R period.

In this method, time is allowed for attainment of equilibrium in the 'plant cell - suspending liquid - gas phase' system before R_1, R_2 and P & R are measured, i.e., these quantities are measured during the steady state.

It is apparent that the true rate of photosynthesis (as μl. O_2 produced per minute) is obtained as follows:

$$\text{Photo.} = \frac{O_{2\,P\,\&\,R}}{\text{Minutes}_{\,P\,\&\,R}} + \tfrac{1}{2}\left(\frac{O_{2R_1}}{\text{Minutes}_{\,R_1}} + \frac{O_{2R_2}}{\text{Minutes}_{\,R_2}}\right)$$

This calculation may be considerably simplified by substituting the h values obtained for the different periods in the following equation:

$$h_{\text{Photo.}} = -\left[\tfrac{1}{2}\left(\frac{h_{R_1}}{\text{Minutes}_{\,R_1}} + \frac{h_{R_2}}{\text{Minutes}_{\,R_2}}\right) - \frac{h_{P\,\&\,R}}{\text{Minutes}_{\,P\,\&\,R}}\right] \qquad (38)$$

where the negative sign before the bracket allows for the correct substitution of either negative or positive values of $h_{P\,\&\,R}$.

Then:

$$X_{O_{2}\text{Photo.}} = K_{O_2}(h_{\text{Photo.}})$$

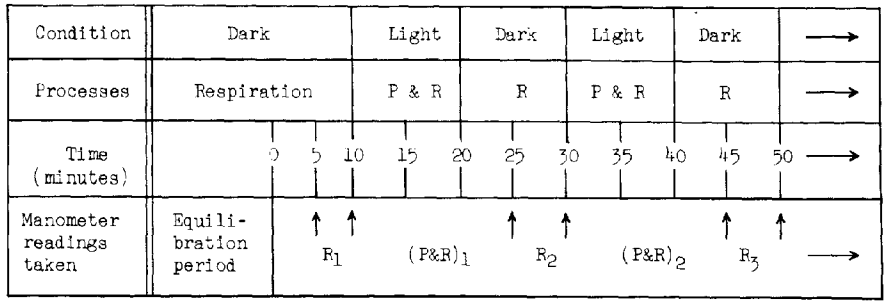
As an example: In a determination of photosynthesis in light of low intensity: h_{R_1}(10 minutes) = -4.0 mm.; h_{R_2}(10 minutes) = -3.8 mm.; $h_{P\,\&\,R}$(10 minutes) = -2.0 mm. (i.e., during the illumination period the rate of respiration was greater than the rate of photosynthesis).

$$h_{\text{Photo.}} = -\left[\tfrac{1}{2}\left((-0.4) + (-0.38)\right) - (-0.2)\right] = 0.19 \text{ mm.}$$

In another case, where high light intensity was used: h_{R_1}(10 minutes) = -2.0 mm.; h_{R_2}(10 minutes) = -2.1 mm.; $h_{P\,\&\,R}$(10 minutes) = +4.0 mm.

$$h_{\text{Photo.}} = -\left[\tfrac{1}{2}\left((-0.2) + (-0.21)\right) - (-0.4)\right] = 0.605 \text{ mm.}$$

B. "Alternating light-and-dark periods" method: The following diagram indicates the general relationships:



As indicated, the rate of respiration is measured during the second 5 minutes of each dark period. The first 5 minutes allows for the attainment of equilibrium conditions of gas exchange for respiration after a period of combined photosynthesis and respiration. On the other hand, while photosynthesis begins as soon as the plant cells receive light and ceases when the light is turned off, the last vestiges of oxygen produced in photosynthesis requires one or two minutes to reach the gas space, and hence it would not be included in the measured oxygen if a manometer reading were taken the instant the light was turned off. The same reasoning holds when the rate of photosynthesis is less than the rate of respiration. Therefore, a manometer reading is taken at the end of the first 5 minutes of darkness. Manometer readings may be continued to be taken in this order for succeeding 10-minute light and dark periods for as long a time as is practical.

The true rate of photosynthesis is calculated as follows: Since respiration has been measured for two 5-minute periods (immediately before and 5 minutes after the photosynthesis-respiration period), the average respiration for the two periods is taken to represent the respiration occurring during each 5-minute period of photosynthesis-respiration. During the 15-minute period of photosynthesis-respiration, photosynthesis occurred for 10 minutes and respiration occurred for 15 minutes. Hence the true rate of photosynthesis for the 10-minute light period is obtained as follows:

$$\text{Photo}_{10\text{ minutes}} = 3\left(\frac{{}^{O_2}R_1 + {}^{O_2}R_2}{2}\right) + {}^{O_2}(P\&R)_1$$

The result can be obtained more quickly by substituting the h values obtained for the different periods in the following equation.

$$h_{10\text{-min. Photo.}} = -\left[3\left(\frac{h_{R_1} + h_{R_2}}{2}\right) - h_{P\&R}\right] \qquad (39)$$

where the negative sign before the bracket allows for the correct substitution of either negative or positive values of $h_{P\&R}$. As an example: In a determination of photosynthesis in light of low intensity: h_{R_2} = -5.1 mm.; h_{R_3} = -5.1 mm.; $h_{P\&R_2}$ = -10.1 mm.

$$h_{10\text{-min. Photo.}} = -\left[3\left(\frac{(-5.1) + (-5.1)}{2}\right) - (-10.1)\right] = 5.2 \text{ mm.}$$

From the above discussion of the methods of measuring photosynthesis by means of the differential manometer, it is evident that in the reaction vessel: (1) both carbon dioxide and oxygen are present in the gas space and are dissolved in the solution in which the plant cells are suspended; (2) during respiration carbon dioxide is added to and oxygen is removed from the gas and liquid phases; (3) during photosynthesis-respiration, if

the rate of photosynthesis exceeds the rate of respiration, oxygen is added to and carbon dioxide removed from the gas and liquid phases; (4) if the rate of photosynthesis does not exceed the rate of respiration during the photosynthesis-respiration period the situation is the same as for (2) above, the rate of exchange only is decreased. In other words, both the oxygen and carbon dioxide content of the gas and liquid phases change. If, during respiration the CO_2/O_2 ratio is 1.0 the net difference in terms of total gas in the reaction vessel is zero; the same is true when the photosynthesis ratio, O_2/CO_2 is 1.0. There would be no change in the manometer reading were it not for the fact that α for oxygen is much less than the α for carbon dioxide. When oxygen is utilized in respiration V_g decreases due to the greater solubility of the carbon dioxide produced in the liquid present. By the same reasoning, V_g increases during photosynthesis.

In extending the theory of the differential respirometer to include this situation: (1) K of the gas space must be determined, since there is no way of determining what proportion of its volume is occupied by oxygen and by carbon dioxide; (2) a means must be found to determine K_{O_2}, i.e., when we know what effect the addition of x amount of carbon dioxide and the removal of the same quantity of oxygen (or the reverse of this exchange) will have on h; (3) how a correction can be made when the photosynthesis ratio differs from 1.0. It does not matter what the respiratory ratio is if photosynthesis is calculated on the basis of the h values for the different periods (see equations 38, 39 above) for what occurs in the reaction vessel is essentially a change in h due to photosynthesis, and this is the only change measured.

(1) Determination of K_{gas} space (K_{gs}): The K desired is the change in manometer reading, h, produced by adding x quantity of gas to the gas space when no gas is absorbed by the liquid present. It is assumed that the volume of liquid occupying V_f (and V'_f) is to be present during an experiment.

The K_{gs} may be determined by using a modification of the simplified equation. $V_f \alpha$ in the third term of the equation, which accounts for the quantity of the gas introduced that dissolves in the liquid, is omitted and the equation used in this form:

$$K_{gs} = h\left[\left(1 + \frac{A\ P_o}{2\ V'_g}\right)\left(\frac{V_g \frac{273}{T}}{P_o} + \frac{A}{2}\frac{273}{T}\right)\right] \qquad (40)$$

where the product of the terms within the brackets is the "gas space constant" of the respirometer. In reality this equation is a variant of the equation derived by Barcroft (1908). The original equation is:

$$K_{gs} = h\left(\frac{273}{T}\right)\left(\frac{V_g\ AP}{P_o}\right) \qquad (41)$$

and was used by Warburg and Negelein (1922) in deriving the constant of their differential manometer. Equation (40) has the advantage of allowing for different volumes of V_g and V'_g. The substitution of experimental values into equations (40) and (41) will yield the same results (within 2%) only if the cross-sectional area of the manometer capillary is less than 0.25 sq. mm.

The constant of the gas space may also be obtained by the Münzer and Neumann method. The general procedure is the same as that described in the section on calibration (p. 54), with the exception that a volume of glass beads equal to the volume of solution which will be used during an experiment is added to each vessel of the respirometer and calcium chloride drying tubes are attached to the end of the manifold and to the gas vents of the respirometer. The use of the calcium chloride drying tubes obviates any correction for the vapor pressure of the water vapor in the air of the room. It is not necessary that V_g and V'_g be adjusted to the same volume. However, the approximate volumes of the flasks can be determined by running in water from a burette, and the difference between V_g and V'_g in microliters of glass beads added to the larger flask, the compensation vessel. The con-

stant is obtained from the h values observed when x volumes of dry gas is added to or withdrawn from the reaction-vessel side of the respirometer,

$$K_{gs} = \frac{x}{h}\left(\frac{273}{T}\right)\left(\frac{P_c}{P_o}\right) \tag{42}$$

If dry gas is not used, a correction for the vapor pressure of water should be applied. In such a case P_c in the above equation is decreased by an amount p_c, which is the vapor pressure of water in the atmosphere of the room in which the calibration is made. If the air is 50% saturated with water vapor at 25°C., this correction reduces K_{gs} by approximately 1.5%.

The complete equation for the Münzer and Neumann method (Eq. 28) can be used to obtain this constant for experimental conditions different from those of calibration.

(2) Determination of K_{O_2} when equal amounts of carbon dioxide and oxygen are exchanged: The extension of the theory to determine K_{O_2} based on the difference in solubilities of carbon dioxide and oxygen in the liquids in the vessels has been described by Warburg and Negelein (1922). It is as follows:

Let: P_o = normal pressure in mm. of manometric fluid.

V_f = µl. of liquid in reaction vessel (also in V').

Y_{O_2} = µl. of oxygen released or absorbed.

Y_{CO_2} = µl. of carbon dioxide released or absorbed.

h = manometer reading, in mm.

$\underline{\alpha}\ O_2$ = absorption coeff. of oxygen at T.

$\underline{\alpha}\ CO_2$ = absorption coeff. of carbon dioxide at T.

V = change in volume of the gas space in the reaction side of the respirometer.

BV = µl. of oxygen added to or removed from the gas space.

(B - 1)V = µl. of carbon dioxide added to or removed from the gas space.

hV = change in the partial pressure of oxygen when the volume of the gas space changes by an amount V.

(h - 1)V = change in the partial pressure of carbon dioxide when the volume of the gas space changes by an amount V.

Then, $V = BV - (B-1)V$

and $h = Bh - (B-1)h$

Thus, $$Y_{O_2} = BV + \frac{Bh(V_f\ \underline{\alpha}\ O_2)}{P_o} \tag{43}$$

and $$Y_{CO_2} = (B-1)V + \frac{(B-1)h(V_f\ \underline{\alpha}\ CO_2)}{P_o} \tag{44}$$

Since $Y_{CO_2} = Y_{O_2}$

on eliminating B and Y_{CO_2} after combining equations (43) and (44)

$$Y_{O_2} = \frac{\left(V + \frac{hV_f\ \underline{\alpha}\ CO_2}{P_o}\right)\left(V + \frac{hV_f\ \underline{\alpha}\ O_2}{P_o}\right)}{\frac{hV_f\ (\underline{\alpha}\ CO_2 - \underline{\alpha}\ O_2}{P_o}} \tag{45}$$

In this equation, h represents the increase in pressure, $\triangle P$, on the reaction side of the respirometer. Actually h is less than $\triangle P$ because of the compensatory effect of the increase in pressure on the compensation-vessel side. A correction may be applied by substituting $\triangle P$ for h, where

$$\triangle P = h + P\left(\frac{V_g}{V_g - \frac{Ah}{2}} - 1\right)$$

This correction reduces the value of Y_{O_2} by about 1%.

Since V is equal to hK_{gs},

$$X_{O_2} = h\left[\frac{\left(K_{gs} + \frac{V_f\underline{\alpha}_{CO_2}}{P_o}\right)\left(K_{gs} + \frac{V_f\underline{\alpha}_{O_2}}{P_o}\right)}{\frac{V_f(\underline{\alpha}_{CO_2} - \underline{\alpha}_{O_2})}{P_o}}\right] \qquad (46)$$

and K_{O_2} is equal to the quantity represented by the bracketed terms in the above equation.

(3) Correcting K_{O_2} when the photosynthetic ratio, O_2/CO_2, is not unity:

Let $\frac{Y_{O_2}}{Y_{CO_2}} = Z$

then equation (45) takes this form,

$$X_{O_2} = h\left[\frac{\left(ZK_{gs} + Z\frac{V_f\underline{\alpha}_{CO_2}}{P_o}\right)\left(K_{gs} + \frac{V_f\underline{\alpha}_{O_2}}{P_o}\right)}{K_{gs}(Z - 1) + Z\left(\frac{V_f\underline{\alpha}_{CO_2}}{P_o}\right) - \frac{V_f\underline{\alpha}_{O_2}}{P_o}}\right] \qquad (47)$$

The above equation is the complete equation to be used with the differential manometer in determining photosynthesis provided the photosynthesis ratio is known. It so happens, however, that a determination of the photosynthesis ratio is not an easy matter. Many workers have either assumed it to be 1.0, or they have used the value obtained by Warburg and Negelein (1922). As a matter of fact, Warburg and Negelein did not determine this ratio under conditions strictly comparable to those which prevailed during their measurement of the quantum efficiency of photosynthesis. They used a glass vessel filled with a gas mixture and a suspension of algal cells, from which samples of gas were withdrawn from time to time and analyzed for oxygen and carbon dioxide. They obtained an average value of 1.1 in three experiments.

It is theoretically possible, but practically impossible to determine the photosynthesis ratio under ideal conditions using the differential respirometer. It would be possible to determine it by the indirect method of Warburg (see Chapter 4), in which the reaction vessels of two respirometers would contain the same volume of cell suspension (and concentration of cells) but with unequal gas spaces; the cell suspensions being exposed to exactly the same temperature and light intensity at the same time. It is doubtful whether any laboratory possesses such a set-up. The nearest approach is to carry out two successive experiments using equi-volume samples of the same cell suspension in flasks of unequal gas volume exposed at the same temperature to virtually identical quantities of light (c.f., Emerson and Lewis, 1941). As indicated above, X_{O_2} and X_{CO_2} can then be obtained by using the indirect method of Warburg, which can be applied directly to the differential respirometer when K_{O_2} and K_{CO_2} are known.

Because of the difficulty of obtaining the photosynthesis ratio (some investigators observe a "burst of CO_2" when algal cells, for instance, are illuminated) and because it effects a simplification of the whole procedure of determining photosynthesis, many workers favor the use of carbonate-bicarbonate CO_2-buffer solutions. Without going into the question of the effect of such solutions on the metabolism of the cell (Warburg, O., 1919; Manning, et al., 1938; Emerson and Lewis, 1942; Pratt, 1943), it is readily understood that if the partial pressure of carbon dioxide in the gas space of the reaction vessel of the respirometer is maintained constant, the change in h is due solely to the oxygen added to or removed from the system by the plant cells. Thus, one need only determine the K_{O_2} for the respirometer containing the particular CO_2-buffer under the conditions of experimentation. There are certain precautions, however, to be borne in mind. In particular: the carbonate-bicarbonate CO_2-buffers change as carbon dioxide is added to or removed from solution; this in turn changes the partial pressure of the carbon dioxide in the gas space of the respirometer vessel. Warburg (1919) has called attention to this fact, and has indicated the working range in terms of the quantity of carbon dioxide that can be removed from or added to the solution without introducing an error of more than 1% in the h values obtained. He also pointed out that the higher the pH of a carbonate-bicarbonate solution the shorter the period of time the plant cells, i.e., Chlorella can remain in it without showing a decrease in photosynthetic capacity. Pratt (1943) has considered this question of the physiological effect of sodium and potassium bicarbonates on the rate of respiration and photosynthesis of Chlorella vulgaris. As a result of his study, he recommends a solution consisting of 0.035 M $KHCO_3$ and 0.065 M $NaHCO_3$. He found that in such a solution the accelerating and depressing actions of potassium and sodium salts, respectively, were balanced and the initial rate of photosynthesis was maintained virtually unchanged for fifteen hours.

Table XVII contains some of the pertinent data for various solutions of sodium carbonate-bicarbonate CO_2-buffers. Solutions of this type were used by Warburg (1919) in determining the effect of carbon dioxide concentration on the rate of photosynthesis in Chlorella.

TABLE XVII

Carbonate-bicarbonate Mixtures

Mixture No.	Composition, mls.		Na millimoles Liter	CO_2 Moles/L 25°C.	pH* 25° C.
	Na_2CO_3 0.1 M	$NaHCO_3$ 0.1 M			
1	85	15	185	0.53×10^{-6}	10.42
2	80	20	180	1.0 "	10.30
3	75	25	175	1.7 "	10.19
4	70	30	170	2.6 "	10.10
5	60	40	160	5.3 "	9.93
6	50	50	150	9.8 "	9.79
7	35	65	135	2.3×10^{-5} "	9.51
8	25	75	125	4.3 "	9.32
9	15	85	115	9.1 "	9.08
10	10	90	110	15.0 "	8.91
11	5	95	105	33.0 "	8.69

*Routine determinations made with a glass electrode.

The data in the above table are presented only to show the composition of some CO_2-buffers that have been used, and to point out the relatively high pH values of such solutions. In case one desires to use such buffers, it is imperative that their effect on the particular process under investigation be determined. It certainly appears that the recommendations of Pratt (1943) should be considered.

Data of an Experiment: The differential respirometer was set up with 36 ml. of algal cell suspension in the reaction vessel (left) and 36 ml. of the nutrient solution without the algal cells in the compensation vessel. Before beginning the actual experiment, the vessels were flushed with 5%-CO_2-air mixture for one hour in order to saturate the liquids with carbon dioxide at this partial pressure. The algal cells were irradiated with red light of low intensity. The shaking of the flasks was stopped momentarily when a reading of the heights of the manometer liquid in the limbs of the manometer was made.

The following data were taken:

TABLE XVIII

Data of a Typical Experiment in Photosynthesis

Period	Time min.	Environment	Manometer Readings, in mm.			
			Left	Right	Difference	Increment
---	0	Light	146.7	146.9	0.2	
	5	"				
	10	"				
	10	Dark				
	15	"	147.5	145.9	-1.6	
R_1						
	15	Dark	147.5	145.9	-1.6	
	20	"	148.0	145.4	-2.6	-1.0
$(P\&R)_1$						
	20	Light	148.0	145.4	-2.6	
	25	"				
	30	"				
	30	Dark				
	35	"	149.4	144.0	-5.4	-2.8
R_2						
	35	Dark	149.4	144.0	-5.4	
	40	"	149.9	143.4	-6.5	-1.1
$(P\&R)_2$						
	40	Light	149.9	143.4	-6.5	
	45	"				
	50	"				
	50	Dark				
	55	"	151.0	142.0	-9.0	-2.5
R_3						
	55	Dark	151.0	142.0	-9.0	
	60	"	151.5	141.6	-9.9	-0.9
$(P\&R)_3$						
	60	Light	151.5	141.6	-9.9	
	65	"				
	70	"				
	70	Dark				
	75	"	152.6	140.4	-12.2	-2.3
R_4						
	75	Dark	152.6	140.4	-12.2	
	80	"	153.0	139.9	-13.1	-0.9

The amount of photosynthesis during the $(P\&R)_3$ period, for instance, can be calculated by the use of equation (39) above. It is:

$$h_{10\text{ min. Photo.}} = -\left[3\left(\frac{(-.9)+(-.9)}{2}\right) - (-2.3)\right] = 0.5 \text{ mm.}$$

This value multiplied by the constant of the manometer is the quantity of oxygen liberated during the 10 minute exposure to light. As is often the case, and as represented above, changes in light intensity from one P&R period to the next result in different values of photosynthesis for the different periods ($(P\&R)_1$, $(P\&R)_2$ etc.).

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Chapter VIII

SPECIAL METHODS EMPLOYING MANOMETRIC AND ELECTROMETRIC TECHNIQUES

In most of the methods listed below some sort of specialized apparatus other than that normally accompanying a respirometer is required. In general, the methods have been devised to meet specific needs not covered by the usual instruments or to enable a measurement which is difficult and time-consuming on the normal instrument, to be done with more convenience. In some instances the method is described in principle only and references to detailed descriptions are cited.

THE "FIRST" METHOD OF DICKENS AND SIMER

In this method oxygen uptake is first determined by absorbing the CO_2 in alkali. At the end of the experimental period, acid is added to the tissue and to the alkali thus liberating all of the CO_2 which can then be measured. A second manometer is used in which all of the CO_2 is so liberated at the start of the experimental period. Hence by this method one can determine:

(1) The oxygen uptake (and its rate) over the experimental period.
(2) The CO_2 liberated over the entire experimental period.

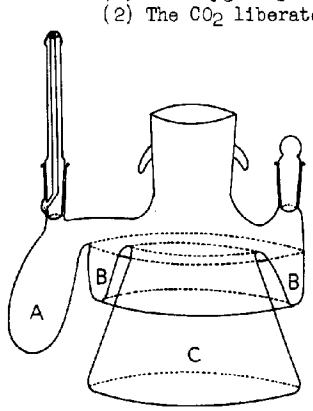

FIG. 26

Flasks for use with the "First" Method of Dickens and Simer. A-sidearm containing acid. B-trough encircling flask containing barium hydroxide. C-main compartment.

Flasks of the type shown in Fig. 26 are recommended, but flasks such as the one shown in Fig. 30 also may be used. Acid is placed in sidearm (A) (0.3 - 0.5 ml. 3N HCl or H_2SO_4). Alkali is placed in the trough (B) (usually 0.5 ml. cold saturated $Ba(OH)_2$ or M/5 to M/10 solutions of other alkalis, relatively free from carbonate). Upon shaking, the alkali swirls about in the trough with little tendency for it to splash over. The respiring tissue is placed in the main part of the flask (C). When flasks as shown in Fig. 30 are used, 0.5 ml. of 4N HCl is placed in the secondary sac of the Siamese sidearm and then 0.5 ml. cold saturated $Ba(OH)_2$, or 2N NaOH relatively free from carbonate, is placed in the primary sac of the Siamese sidearm; a folded piece of filter paper is added to increase the absorptive area of the alkali. A substrate can be added from the simple sidearm of the flask. The acid is tipped from the secondary to the primary sac of the Siamese sidearm and thence into the main chamber to obtain the initial bound CO_2 or at the completion of the run to obtain the CO_2 evolved during the run.

The method is quite useful but has the following limitations:

(1) Bicarbonate solutions cannot be used because the measurements are made in the absence of CO_2 in the air.
(2) The tissue is respiring in the absence of CO_2, which may affect the rate or course of metabolism.

Detailed descriptions are given by Dixon (1943), Dickens and Simer (1930, 1933) and Meyerhof and Schmitt (1929).

THE "SECOND" METHOD OF DICKENS AND SIMER

The basic principle of this method is that after an appropriate interval acid is added to the tissue liberating all bound CO_2 in the form of gaseous CO_2. The CO_2 in the

gas phase is then all absorbed by the addition of alkali. Appropriate controls are used which permit one to measure oxygen uptake, acid production, and CO_2 production in buffers, bicarbonate, serum, etc., or mixtures of these. The actual description of the method is quite complex but it is certainly desirable to have some knowledge of the principles involved, since when equipment is available permitting its use, the method eliminates many of the complexities and uncertainties inherent in other methods, although it does introduce a few of its own.

The essential measurements are the CO_2 liberated by acid and the CO_2 absorbed by alkali. Two types of apparatus are available for this measurement. First the type of flask used by Dickens and Simer will be described. This is a two compartment flask illustrated in Fig. 27, fitted with sidearms, one of which tips into one compartment (i.e., A' into A); the other tips into the second compartment (B' into B). The tissue is placed in

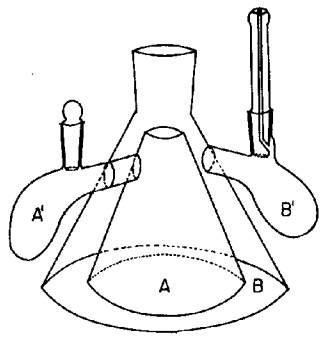

FIG. 27

Flask for use with the "Second" method of Dickens and Simer.

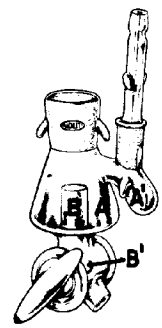

FIG. 28

Dixon-Keilin Flask (see text for description.

compartment A and acid in A'. Upon tipping A' into A the CO_2 fixed in the solution is released. Compartment B contains M/5 $KMnO_4$ in M/500 H_2SO_4 while the sidearm (B') contains 30% NaI (acidified to M/500 H_2SO_4 just before use). Upon tipping B' into B mixing of the iodide with the permanganate results in an alkaline reaction which absorbs the whole of the CO_2 from the gas phase.

A second type of flask which accomplishes the same purpose is that used by Dixon and Keilin, illustrated in Fig. 28. Tissue is placed in compartment A', and acid in the sidearm A'. Alkali is placed in the stopcock insert B' which when turned to a point parallel with the center well (B) permits the alkali to enter the flask. "B" usually contains filter paper to increase the surface of the alkali. A glass rod, by dropping into the stopcock insert displaces the alkali into B so that it wets the paper.

Measurement of respiration, CO_2 output and acid production are made with one flask but two others are required as controls. Details of the procedure have been adequately described in several places (Dixon, 1943; Dickens and Simer, 1933); the method is briefly as follows: In the center compartment of the principal flask, the tissue is suspended in phosphate, in other buffers, or in serum and is allowed to respire for a definite measured interval. At the end of this interval acid is tipped in from the sidearm, and the gas so released (CO_2) measured (= final "bound" CO_2). After the readings are constant, the iodide is tipped into the permanganate (or alkali added in the Dixon-Keilin flasks) causing the absorption of CO_2. This is measured (= final CO_2 in gas phase). Oxygen uptake is determined by the drop in the reading after all the CO_2 has been absorbed compared to the

initial reading. This drop was caused by (a) oxygen uptake, and (b) the absorption of the CO_2 which was in the gas phase at the start. A control flask is used to determine how much CO_2 was in the gas phase at the start, hence the oxygen uptake can be calculated readily. The CO_2 in the gas phase not accounted for by the initial CO_2 in the gas, plus that derived from the bicarbonate (a second control flask measures initial "bound" CO_2) is that produced directly by respiration or fermentation. The decrease in bicarbonate is a measure of the acid production, but it must be corrected for "acid retention" (see later) in order to give the true acid produced. The relatively large changes in gas pressure which occur (i.e., when bicarbonate is acidified, or all the CO_2 is absorbed) usually necessitate the use of mercury or Clerici's solution in the manometer. While the method is quite useful it has two limitations:

(1) "Acid retention" must be estimated and corrected for. (See discussion in the latter part of this chapter.)
(2) It only serves to measure the reactions over an interval and does not provide for a continuous measurement of respiration, CO_2 output and glycolysis, as has sometimes been supposed.

Undoubtedly this method deserves more attention than has been accorded to it. In our opinion the necessity of specialized apparatus has been a serious limitation in its use. A modification which permits one to use the method with ordinary Warburg flasks having two sidearms is the following:

Tissue, buffers, etc., are placed in the main compartment. Acid is placed in one sidearm. The other sidearm usually contains 0.3 ml. saturated $KMnO_4$ in M/1000 H_2SO_4. Suspended above it is a tube of KI (solid) attached to the stopper with sealing wax (Fig. 29, see Vogler, 1942). When, after the acid has been added, it is desired to absorb the CO_2 from the gas phase, the stopper is turned to break off the tube containing the KI; the KI mixes with the permanganate and causes an alkaline reaction whereupon the CO_2 is absorbed from the gas phase. The absorption is slow, however, and 8-12 hours are required for completion. This does not seriously affect the use of the method since the tissue is not metabolizing. The manometers are usually left in the bath (shaking slowly) overnight. Mixing of KI and $KMnO_4$ also can be accomplished by the use of a flask having one of its sidearms equipped with a double sack (Fig. 30). This type of flask (Fig. 30) is less expensive, more rugged and suitable for a wider variety of applications than the more specialized flasks shown in Figs. 26, 27, 28; it is highly recommended as a general utility flask. As these flasks occupy more space than the ordinary flasks it may be necessary to stagger them in the bath, i.e., if the sidearms of one flask point away from the manometer the sidearms of the adjacent flask will point toward the manometer.

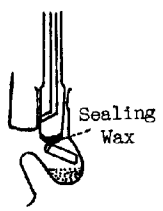

FIG. 29

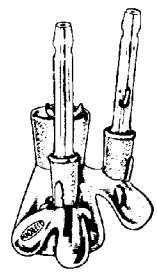

FIG. 30

Arrangements permitting the mixing of two materials in a sidearm (see text for description).

DIXON-KEILIN METHOD

This method is adequately described by Dixon (1943) together with several modifications. It is essentially the second method of Dickens and Simer but has been considerably simplified for use with the differential manometer. When this apparatus is available it is undoubtedly a very excellent method. Two flasks (Dixon-Keilin type) of exactly the same size (their size may be equalized by adding glass rods (Summerson, 1939)) and containing identical amounts of tissue, buffers, gas, etc. are attached to opposite sides of a differential manometer. At the start, acid is tipped into one of the flasks, giving the initial "bound" CO_2. Respiration, glycolysis, etc. continue in the other flask, causing a drop in the manometer reading. At the end of a measured interval acid is tipped into the second flask causing the liberation of "bound" CO_2 still remaining. Both flasks have

now had the same treatment except that one has respired for a measured interval. Alkali is now introduced into both flasks simultaneously to absorb the CO_2 in the gas phase. From the resulting change in the manometer, the oxygen uptake can be determined readily. From the other readings mentioned one may determine the total CO_2 output and the change in "bound" CO_2. From the latter, by correcting for "acid retention", the acid produced may be calculated. Dixon (1937), Brekke and Dixon (1937), Elliott and Schroeder (1934) have described methods based on the Dixon-Keilin method. Dickens and Greville (1933b) and Summerson (1939) have provided essentially similar methods but with improvements possible by the use of specialized apparatus. Dixon (1943) has discussed these in some detail.

W. W. Umbreit

RETENTION

The measurement of CO_2 production is complicated by retention. Retention is a manifestation of the fact that the amount of CO_2 released from a buffered medium is less than would be released from a theoretical medium with no buffering capacity. Quantitatively, retention by a medium is a direct function of its buffering capacity.

Two manifestations of retention are encountered experimentally, and they will be discussed here under the terms "retention of CO_2" and "retention of acid". The difference between them is that "retention of CO_2" concerns the binding of a part of the metabolic CO_2 (determined as outlined in Chapter 2) as bicarbonate by reaction with the buffer, and "retention of acid" concerns the neutralization of a part of the metabolically formed acid (determined as outlined in Chapter 3) by the buffer present rather than by bicarbonate.

Retention of CO_2. When metabolic CO_2 is produced in a medium in the usual physiological range of pH, less CO_2 appears in the gas phase than is predicted by the equation for the measurement of CO_2 exchange: $x_{CO_2} = hk_{CO_2}$. The flask constant $k_{CO_2} = \dfrac{V_g \frac{273}{T} - \alpha V_f}{10{,}000}$ is calculated with the absorption coefficient, α, for CO_2 as one factor. The values for α substituted in the equation are determined from the solubility of CO_2 in pure water, i.e., in a medium essentially without buffering capacity. The true absorption coefficient, α, is not affected greatly by a change in pH or by the presence or absence of buffers in the medium, e.g., the α value for CO_2 at 25° C. is 0.756 in water, 0.732 in 1.0 M HCl, and 0.692 in 1.0 M NH_4Cl. However, as was emphasized in Chapter 3, the observed solubility of CO_2 is complicated by the formation of bicarbonate in a medium with buffering capacity. Retention of CO_2 is the term used to describe the ability of a medium to bind CO_2 (as carbonate or bicarbonate) in excess of the amount predicted by its true α solubility.

Correction for retention of CO_2 is made most easily by using a flask constant derived by substituting an α' value for α in the equation for determining the flask constant. α' is defined here as the solubility of CO_2 at the pH of the medium, and it combines both the true α solubility and the solubility from the formation of bicarbonates. As pK'_a from the apparent first dissociation constant for carbonic acid is 6.317 (at 38° C.),

$$\frac{\alpha'}{\alpha} = \frac{(HCO_3^-) + (CO_2)}{(CO_2)} = \left[\text{antilog } (pH - 6.317)\right] + 1$$

where the pH is that in the reaction flask during the experiment. In Fig. 7, Chapter 2, $\frac{\alpha'}{\alpha}$ is plotted against pH. An inspection of this figure indicates that below pH 5 retention is negligible and may be disregarded, that in the pH range 5 to 7 retention is considerable but may be corrected for with the anticipation of accurate results, but that retention above pH 7.0 is excessive and accuracy may be poor when the "direct method" for measuring CO_2 production is used.

As an example of the use of the α' value, assume that a flask of 18.5 ml. volume is being used to measure the CO_2 production by cells suspended in 3 ml. of medium at pH 6.5. The temperature of the bath is 37° C. Fig. 7 indicates that at pH 6.5 $\frac{\alpha'}{\alpha} = 2.50$. At

37° C. the value of α for CO_2 is 0.567, so $\alpha' = 2.5 \times 0.567 = 1.42$. Therefore,

$$k'_{CO_2} = \frac{15{,}500 \times \frac{273}{310} + 3{,}000 \times 1.42}{10{,}000} = 1.79.$$

Under the conditions specified the true CO_2 production can be calculated simply by multiplying the observed change in mm. on the manometer (Brodie's fluid) by 1.79.

The use of α' in correcting for retention carries with it the assumption that the pH in the reaction vessel does not change. If the pH does change the final as well as the initial pH should be determined to permit a more accurate calculation of the CO_2 retained. As reactions proceeding at a linear rate are normally measured, a knowledge of the initial and final pH will permit the reasonably accurate prediction of the pH at any time during the run; from the pH at any time the α' and the flask constant at that time can be calculated. In many cases the buffering capacity of the medium will be sufficient so that changes in the pH may be neglected.

Although the use of α' values for the calculation of retention of CO_2 is recommended for its simplicity, it also is possible to determine the retention empirically. This is done by generating CO_2 in a respirometer in the absence of the medium and in another respirometer in the presence of the medium. The procedure usually recommended is to place bicarbonate for generating CO_2 in the flasks (section B, Fig. 27, or primary sac of Siamese sidearm, Fig. 30) and to fill the flasks with a gas mixture containing CO_2 in equilibrium with the bicarbonate. A simpler procedure is to substitute carbonate for bicarbonate so the determination may be performed in an atmosphere of air. The details of such a determination follow:

Flask 1 (A flask with 1 sidearm)	Flask 2 (A flask of the type shown in Figs. 27 or 30)
0.2 ml. solution containing 1 mg. Na_2CO_3 (equivalent to 211 μl. CO_2) is dried in main chamber of flask at 250° C. for 1 hour.	0.2 ml. solution containing 1 mg. Na_2CO_3 is dried in primary sac of Siamese sidearm (Fig. 30) or in chamber B (Fig. 27) at 250° C. for 1 hour.
0.2 ml. 0.2 N H_2SO_4 in sidearm. (This is more than sufficient to liberate all the CO_2 from the Na_2CO_3.)	0.2 ml. 0.2 N H_2SO_4 in secondary sac of Siamese sidearm (Fig. 30) or in B' (Fig. 27).
2.0 ml. H_2O in main chamber.	2.0 ml. of medium in main chamber (Fig. 30) or in A (Fig. 27).
Atmosphere, air.	Atmosphere, air.

After equilibration, tip in acid from the sidearm into the main chamber of flask 1. In flask 2 tip acid from the secondary into the primary sac of the Siamese sidearm (Fig. 30) or from B' into B (Fig. 27). The addition of acid liberates the same amount of CO_2 in each flask, but more is taken up by the medium in flask 2 than by the water in flask 1. The amount of CO_2 liberated into the atmosphere of each flask is calculated from the observed change in pressure (corrected for any change in the thermobarometer) and the k_{CO_2} for each flask. As the same amount of CO_2 was liberated from the carbonate in each flask, the CO_2 observed in flask 1 minus that observed in flask 2 represents the retention of CO_2 by 2 ml. of the medium (the α solubility in the fluid in the flasks is corrected for in the calculation with k_{CO_2}). If, for example, only 80% of the CO_2 liberated is observed in the gas phase, all observed values for CO_2 liberation under the same conditions can be divided by 0.80 to give the true amount of CO_2 formed.

It should be noted that in the example of the empirical determination of retention of CO_2 only the medium is considered and no enzymatic preparation is included in the test. When a homogenate or other preparation with considerable buffering capacity is added, the retention is changed substantially. Addition of heat inactivated enzyme, or enzyme plus inhibitor, to the medium for the test of retention capacity should correct for this, although denaturation and clumping of the protein upon heating may alter its buffering capacity somewhat.

Retention of Acid. Production of acid often is followed manometrically by measuring the CO_2 released by the acid from a bicarbonate medium. However, the acid produced does not release its equivalent in CO_2 because of the buffering capacity of the medium.

An electrometric titration curve of materials duplicating those contained in the reaction flask and a determination of the initial and final pH in each flask will permit the calculation of retention of acid. However, it is difficult to obtain accurate pH values because of the loss of CO_2 from the medium after the vessel is opened. The pH values could be found by inserting a glass electrode and bridge assembly through a ground joint in the flask, but this would require special equipment.

The empirical determination of retention of acid (especially by the method described by Bain in the next section) is the method of choice for obtaining a correction factor. A known amount of acid, insufficient to liberate all of the CO_2 from bicarbonate, is added to bicarbonate in one flask and to bicarbonate plus the medium in another flask; the output of CO_2 is measured in each case. Note that in this method the acid is added directly to the medium, whereas in the empirical determination of retention of CO_2 it is added to the carbonate in a chamber separated from the medium. An example of such a determination, carried out in ordinary flasks with one sidearm, follows:

Flask 1	Flask 2
Main chamber - 1 ml. 0.0177 M $NaHCO_3$ 2 ml. water	1 ml. 0.0177 M $NaHCO_3$ 2 ml. medium
Sidearm - 0.1 ml. 0.040 M lactic acid	0.1 ml. 0.040 M lactic acid

Gas mixture contains 5% CO_2; pH of medium 7.0.

After equilibration, acid is rinsed into the main chamber of each flask. The amount of CO_2 liberated is calculated from the change in the manometer readings (corrected for any change of the thermobarometer) and the flask constants. The μl. CO_2 liberated in flask 2 divided by the μl. CO_2 liberated in flask 1 gives the fraction liberated from the medium. If this be 0.8, subsequent values for CO_2 liberation, from this medium under the same conditions, when divided by 0.8 will yield the true amount of CO_2 equivalent to the acid produced.

The method described involves the addition of a constant amount of acid. If it is added in flasks of different volumes it will not establish the same pCO_2; therefore, retention of acid will not be the same in flasks of different volumes. Hence, calibration of each flask is necessary; this is a cumbersome operation when accomplished by the procedure outlined above. In the following section a very convenient method for determining retention of acid during a run is described.

R. H. Burris

MEASUREMENT OF RETENTION OF ACID

Warburg flasks, so designed as to allow the addition of two substances to the system at different times are required. Flasks with two sidearms were found to be convenient. In one sidearm is placed a measured amount of standard citric acid (0.1-0.2 ml. M/10). The flask is then placed in an oven at 75° C. until the acid is completely dried. This is

done in order to avoid changes in volume when the standard acid is added to the system. In the other sidearm is placed the requisite amount of substrate solution while the main part of the flask contains the tissue to be studied, the $NaHCO_3$ buffer, and water to make a total volume of 3.0 ml. The flask is gassed and equilibrated in the usual manner and the substrate tipped in. The rate of CO_2 evolution is measured for two five minute periods; at the end of the second period the dried acid is washed into the main part of the flask and three more readings are taken. The first two and the last two readings give the steady rate of the system. The difference between this rate and the third reading gives the amount of carbon dioxide evolved by the standard acid. The variation between this value and the value obtained by tipping standard acid into $NaHCO_3$ alone allows a calculation of the retention correction to be made. The amount of acid introduced into the system is not large enough to change the pH appreciably and thus the activity of the tissue is not affected. The correction obtained is a function not only of the buffering capacity of the medium, but also of the volume of the flask and the amount of the gas evolved.

In the conventional method (Dixon, 1943) each flask is calibrated individually. A method has been devised whereby any number of flasks may be calibrated for retention from the data obtained by the use of just one flask provided the volume of each is known.

If the flask constants (k) are calculated for a series of flasks in the usual manner and plotted against the gas volumes (Vg) a straight line, hereafter referred to as the base line, is obtained. Suppose that a medium which retains carbon dioxide is introduced into these flasks. Each flask will now have a constant which is equal to k plus an amount "r" which will vary with each flask. If these new k values be plotted against V_g as before, a straight line will again be obtained, but will be found to lie above the base line and to have a different slope. The characteristics of this line are reflections of the facts that the amount of retention is a function of the buffering capacity of the medium, the volume of the flask, and the amount of gas evolved.

By introducing a given medium into three flasks of different volumes and measuring the amount of CO_2 given off when a known amount of standard acid was added, a retention line was determined directly. This was done with several media of different retentions and the data plotted (see Bain and Rusch, 1944). It was found that the slope (m) of these lines was proportional to the value of k at a given volume, i.e.:

When V_g is constant:

$$k_I m_{II} = k_{II} m_I \qquad (52a)$$

$$k_I m_{III} = k_{III} m_I \qquad (52b)$$

$$k_I m_{IV} = k_{IV} m_I \qquad (52c)$$

With this fact established it became apparent that the retention line for a given medium could be determined from the data of one flask if the base line and the volume of the test flask were known. The method of arriving at this conclusion is demonstrated as follows:

From Chapter 1, $k = x/h$, where k = the constant of the test flask of volume V_g, x = the theoretical µl. of carbon dioxide evolved by n ml. of standard acid, and h = the manometer reading produced by n ml. of standard acid.

Solving the established proportion for m, we find $m = km_{base}/k_{base}$, where m is the slope of the retention line, m_{base} the slope of the base-line and k and k_{base} the constants of the flask of volume V_g at retention and at base-line levels.

By solving a simple analytical equation for a straight line, we find $k' = m(V'g - Vg) + k$, where k' is the constant of the flask of volume V'g at retention line level.

With V_g known and V'g arbitrarily assigned, k and k' can be calculated from the above equations and the retention line (thus defined by the points (k, Vg) and (k', V'g)) con-

structed. The constant for any apparatus volume may now be read directly from the retention curve for the medium involved.

The use of these retention values in the Warburg "indirect method" (Chapter 4) is described by Dixon (1943), in the second method of Dickens and Simer (Chapter 8) by Dixon (1943) and Dickens and Simer (1933), and in the Dixon and Keilin method (Chapter 8) by Dixon (1937, 1943).

J. A. Bain

MEASUREMENT OF GASES OTHER THAN OXYGEN AND CARBON DIOXIDE

Hydrogen: Hydrogen exchange often may be involved in bacterial reactions. In the oxidation of hydrogen with molecular oxygen (the Knallgas reaction) a combination of oxygen and hydrogen takes place and difficulty arises in establishing how much of each gas reacts. If the reaction can occur at low partial pressure of oxygen the individual gases taken up in the overall reaction can be measured as described by Lee, Wilson, and Wilson (1942). Employing a mixture of 98% H_2 and 2% O_2 in the Warburg flasks they allowed the reaction to proceed to a point where the oxygen was exhausted. The oxygen supplied was accurately measured in independent flasks containing pyrogallol and alkali, the alkali being added from the sidearm after temperature equilibrium was reached. Such a method is limited to gas mixtures containing not over 2.5% of oxygen. Subtracting the pressure change in the oxygen analysis flask (alkaline pyrogallol) from the total pressure change in the flask containing H_2 and O_2 indicated the pressure change attributable to H_2 uptake.

If hydrogen evolution is to be measured in an anerobic system for which independent analysis has demonstrated that hydrogen and carbon dioxide are the only gaseous products, the carbon dioxide may be absorbed with KOH in the inset cup and the gas pressure attributed to hydrogen production. Woods and Clifton (1937) have followed such hydrogen output with simultaneous estimation of carbon dioxide evolution in independent flasks.

Nitrogen: In the biological reactions of nitrogen fixation and denitrification molecular nitrogen is involved. The amounts of N_2 reacting are usually relatively small, but can be measured by manometric methods.

Nitrogen fixation may be followed directly in Warburg respirometers in the following way: The Brodie's fluid in the manometers is replaced with mercury which has been moistened with water; 1 to 5 mm. of water is kept above each mercury column to minimize the sticking of the mercury in the capillary tubes of the manometers. The biological agent to be studied is introduced into a Warburg flask arranged so the alkali may be mixed with pyrogallol at the end of the run (flasks shown in Figs. 26, 27, 28 and 30 are applicable). The cultures are shaken in the Warburg bath in the ordinary manner, and pure oxygen is added to the flasks when needed as indicated by the manometer. If impure tank oxygen is used the amount supplied must be recorded so a final correction may be made for N_2 added with the O_2. After it is judged that the biological agent has accomplished its nitrogen fixation, the alkali which has absorbed CO_2 in the vessel is mixed with the solution of pyrogallol (slightly acidified to limit spontaneous oxygen absorption) and the manometer is shaken until equilibrium is reached. The absorption of oxygen will yield the percentage of oxygen initially present in the flask if no nitrogen has been fixed, however, if nitrogen has been fixed its removal will appear as an apparent increase in the oxygen content of the flask. For example, if the gas mixture initially supplied were air with 21% oxygen, and the final absorption of oxygen indicated that 23% of the gas supplied had disappeared, one would conclude that 2% of nitrogen had been fixed. Such measurements may prove useful in establishing nitrogen fixation by materials which by virtue of a high initial nitrogen content cannot be accurately analyzed with the Kjeldahl procedure. The method has been applied by Allison, Hoover and Minor (1942) and in our laboratories; the theory of the method as used with Novy-Soule respirometers is discussed by Hurwitz and Wilson (1940).

Burk (1934) demonstrated that nitrogen fixation could be followed indirectly by estimating the growth rate of nitrogen fixing organisms as measured by oxygen uptake. In a nitrogen free medium the growth of azotobacter is limited by its ability to fix nitrogen. The more rapidly the organism fixes nitrogen the more rapidly it multiplies and in turn

the more rapidly it takes up oxygen. Thus a measurement of the increasing rate of oxygen uptake gives a close approximation of the rate of nitrogen fixation although the two reactions are not strictly parallel.

R. H. Burris

MISCELLANEOUS METHODS

Vogler method: Vogler (1942) described a method for studying CO_2 fixation. This method is essentially that of Dickens and Simer (1933) except that Dixon-Keilin flasks were used and, at appropriate intervals, gases were added. The amounts of gas (CO_2) added were measured manometrically by introducing them into the sidearm and measuring the increased pressure resulting from such addition. A few moments are required before gases added to the sidearm diffuse into the main compartment where they react and this time is sufficiently long to permit accurate estimation of the amounts added. Under acid conditions Vogler was able to estimate CO_2 fixation and O_2 uptake simultaneously.

Gaffron method: In measurements of simultaneous oxidation of hydrogen and reduction of carbon dioxide, Gaffron (1942) has described in detail two methods for absorbing carbon dioxide after the reactions were complete. One of these employed flasks in which the sidearm is attached, not to the body of the flask itself, but to the ground glass joint by which the flask is attached to the manometer. The manometer connection itself has been partially cut away so that by rotating the flask in one direction, the sidearm becomes part of the gas space of the flask, whereas by a further rotation this arm may be closed off (see Fig. 31). There are some minor technical difficulties associated with the use of this type of system. For example, when the sidearm is closed off, pressure changes in the flask have no effect on this isolated gas space, hence upon connecting the sidearm with the main compartment a sudden equalization of pressure occurs between them. Yet this method is one of the few systems available in which one can successively expose tissues to CO_2-free gas and to gas with CO_2 (or other gases for which specific adsorbants are available).

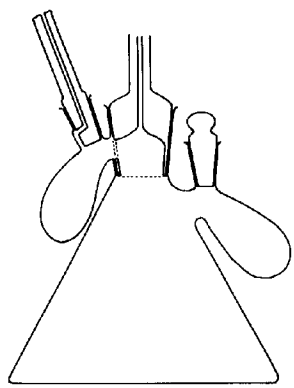

FIG. 31

Flask employed in the Gaffron method.

Another method employs the usual type of flask with a glass paddle fused to the plug of the sidearm. In this sidearm is placed a small thin-walled glass bulb filled with KOH solution. Upon turning the plug of the sidearm the paddle is rotated so that it crushes the bulb, thus releasing the KOH which absorbs the CO_2.

Gas analysis: Two general types of procedure are employed in using the Warburg instrument as an instrument of gas analysis. For gas mixtures containing less than 2 to 2.5% of the gas to be measured, the flasks are filled with the gas to be analyzed (either by the flow method or the evacuation method, see Chapter 5). A reaction is then caused in the flask (or a reagent added) which will absorb the gas. The decreased pressure observed is a measure of the gas content. This method has been used for oxygen (see page 83) by using slightly acidified solutions of pyrogallol in the main compartment and tipping in alkali from the sidearm after the system is equilibrated. It has been used for CO_2 by generating alkali by means of permanganate and iodide (see page 78) or by using Dixon-Keilin flasks (e.g., see Vogler, 1942).

The second procedure, for gas mixtures containing more than 2 to 2.5% of the gas to be measured, is as follows: the reagent for absorbing the gas is placed in the main compartment of the flask and the flask filled with an inert gas. After equilibrium is obtained, the gas to be analyzed is introduced into the sidearm. The increased pressure, while the gas remains in the sidearm, is a measure of the total amount of gas introduced. As the gas diffuses into the main compartment the reagent absorbs it and the resulting

decrease in pressure is a measure of the specific gas involved. Three methods of adding the gas to be analyzed may be used, as follows:

1. Addition to sidearm: Sidearm flasks equipped with a gas vent are employed (Fig. 13, page 44). Before adding the reagent to the flask, the gas is allowed to flow through the sidearm plug until all the original gas in it has been displaced by the mixture to be analyzed. The reagent is added to the flask, the sidearm plug inserted (closed position so that there is no flow of the gas into the flask; reservoir remains attached to plug), and the gas in the flask is replaced with an inert mixture by evacuation procedure (see page 44). After equilibration (during which time the reagent absorbs any of the gas in question which may have entered the flask while inserting the sidearm plug), the plug is turned momentarily to allow gas to enter the flask. The increase in pressure is measured (giving the µl. of gas added). After the gas has diffused from the sidearm and been absorbed by the reagent, the pressure change may be used to calculate the composition of the gas added.

2. Addition to sidearm: This is essentially the same as above, except that the sidearm plug is filled with the gas mixture in question by evacuating and refilling with the gas mixture and is left attached to the gas reservoir. Several (4 or 5) evacuations and refillings are necessary.

3. Addition through manometer stopcock: Gas also may be added through the stopcock of the manometer after flushing out the connecting tubing through the tail-vent of the three-way stopcock. This is an especially convenient method of gas addition. However, the gas tends to remain in the capillaries for a long time. It may be forced into the flask by closing off the open end of the manometer and raising the fluid until it travels along the capillary connecting the manometer to the flask, but even then there is a "dead" space between the flask connection and the stopcock. This method is useful when great accuracy is not required.

The reagents one employs for absorbing gases, depend upon the nature of the gas. Standard works on gas analysis should be consulted for their preparation.

Disinfectants and Germicides: The action of killing agents may be studied manometrically. In principle these methods depend upon adding the toxic agent to the metabolizing tissue or bacterial suspension and determining the amount of inhibition (of respiration or glycolysis) under specified conditions. See Bronfenbrenner, et al. (1938) and Ely (1939) for details.

Catalytic Hydrogenation: The Warburg respirometer can be used for the quantitative study of many catalytic hydrogenations. These take place at 1 atmosphere pressure even though on a larger scale several atmospheres of pressure may be necessary. Apparently the relatively enormous quantity of catalyst in the Warburg vessel in relation to the material to be reduced permits more efficient operation of the catalyst, and hence higher pressures are not usually required. The procedure is useful to determine how much hydrogen is being absorbed but is not intended to actually prepare the products of hydrogenation. Very small quantities of substances can be used and the general conditions for optimum reduction worked out before progressing to large scale reduction. The process is illustrated by an example: In a Warburg flask was placed 1.5 ml. 0.1 M borate buffer, pH 9, 0.5 ml. of a suspension of platinum black containing 1 mg. platinum per ml. In the sidearm was placed 0.2 ml. of a pyridoxal hydrochloride solution containing 5 mg. per ml. (m. w. = 204, hence the 1 mg. added = 4.9 µM) and 0.3 ml. of the borate buffer. Hydrogen gas was passed through the flask for 1 hour, after which the taps were closed and the slight residual uptake of hydrogen (required for the complete reduction of the catalyst) allowed to proceed to completion. The pyridoxal hydrochloride was then tipped in, and in 20 minutes 114 µl. of H_2 was taken up; at this time the reaction stopped. This represents 114/22.4 = 5.1 µM H_2 or 1.04 H_2 per mole of pyridoxal and indicates reduction of the free aldehyde group but no reduction in the ring. Upon using the same reagents but adjusting the buffer to pH 5, 2.63 H_2 per pyridoxal were absorbed indicating direct reduction of the pyridine ring. This method has not been used extensively by organic chemists for the quantitative measurement of hydrogenation, but it is convenient and worthy of more general application. One obvious precaution should be added - the buffer used must not be reducible in the presence

of the catalyst. Phthalate buffers, for example, cannot be employed with most catalysts. The catalysts, such as platinum, palladium, Raney nickel, etc., are prepared in the same manner as for large scale reductions.

W. W. Umbreit

IMPROVED CONSTANT VOLUME RESPIROMETER

A modified design of the conventional Warburg manometer has been developed which permits a 50% increase in range and easier manipulation with a slight decrease in overall dimensions. The changes involved (Fig. 32) are as follows:

1. The fluid reservoir is placed in the right limb of the manometer near the base.
2. The left limb is extended to the bottom of the mount, the space formerly occupied by the reservoir being used for extention of the graduated scale to a total of 45 cm.
3. The unused graduations on the right limb are replaced by a single scratch mark. Further marks may be made at any convenient point.
4. The stopcock is replaced by a standard capillary T 3-way cock placed between the manometer and the flask. The middle tube of the cock is bent forward to protrude slightly between the manometer limbs for gassing purposes.

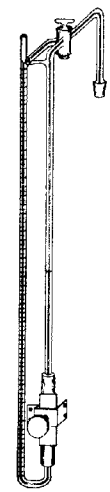

FIG. 32

Sketch of the McGilvery-Machlett manometer, showing method of mounting fluid-level control.

The manometer is calibrated and read in the usual manner. The sensitivity is equal to that of the conventional manometer using the same fluid. Gassing may be accomplished in any of three ways:

a. The cock is turned to connect all three arms, and the same procedure is used as with the conventional manometer.

b. The gas above the manometer fluid is flushed either by moving the fluid up and down while circulating gas through the cock, or by running the fluid up to the cock and turning the latter to connect only the flask and gassing tube. This may be performed at any convenient time - hours before the experiment is to be run, if desired. The flask is then flushed with gas, which may be run through at any pressure without disturbing the manometer column.

c. The manometer is flushed as in b, and the flask is alternately evacuated and filled with the desired gas mixture as described for the conventional apparatus (Chapter 5). No clamps for the fluid reservoir are necessary during the evacuation since the manometer is sealed off from the flask.

At the conclusion of a, b or c, the cock is turned to connect the flask and manometer, and the experiment is continued as with the conventional apparatus.

The design is especially convenient when the manometer is used with the mounts and circular gassing rack employed with the Lardy circular bath. The position of the stopcock on top of the mount permits easy access from either front or back, and a short length of tubing serves to connect the gassing manifold to the gassing tube of the manometer without interference with stopcock manipulation.

The manometer may be placed on the manometer mounts for either rectangular baths or the improved circular bath by making two changes. A hole is drilled in the top of the manometer mount to accommodate the stopcock, and the metal bracket for the fluid level control is moved to the new position of the reservoir.

The manometers may be obtained from E. Machlett and Son, 220 E. 23rd Street, New York, New York, who have been authorized to produce the design. The mounts are supplied as an

optional part of the GME-Lardy circular equipment. (GME, 4 Franklin Avenue, Madison, Wisconsin.)

R. W. McGilvery

OTHER TYPES OF RESPIROMETERS

Two types of constant-volume differential manometers have been devised which combine the advantages of the "Warburg" constant volume type and the "Barcroft" differential type. The first of these, the Dickens and Greville (1933a) instrument is illustrated diagrammatically in Fig. 33. The volume is maintained at a constant value by adjusting the height of the manometer arms so that the fluid in the manometer remains at a constant mark. The difference in fluid levels in the two arms is read from a graduated scale. This permits one to employ a compensated system, independent of changes in barometric pressure, yet using the simple flask constants of the Warburg instrument. The second instrument, that of Summerson (1939) is illustrated in Fig. 34. It is an extremely adaptable instrument, being

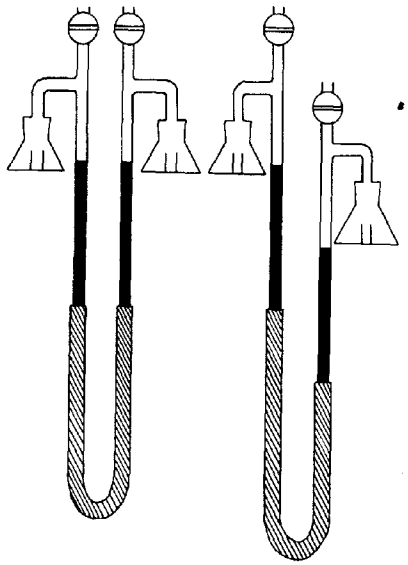

FIG. 33

Diagram of the Dickens-Greville Constant-Volume Differential Manometer.

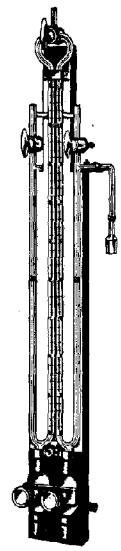

FIG. 34

Summerson Manometer and Support.

capable of serving as two ordinary Warburg manometers (which indeed comprise the main portion of the apparatus) or of serving as a constant volume differential manometer in which case the two outer columns, attached to the flasks, are held constant and the difference in reading between the two inner columns is noted. There is little doubt that this is a very useful instrument, but at present it is not widely distributed.

Sometimes it is desirable to measure larger quantities of gas exchange than can be handled with the standard Warburg instrument. Macrorespirometers involving essentially the same principles but employing larger flasks may be used. Suitable modifications have been described by Wells (1938) and Wood, et al. (1940).

Several types of ultramicrorespirometers have been developed for measuring less gas exchange than can be measured accurately with the Warburg instrument. Certain of these have been described in some detail by Dixon (1943), Perkins (1943) and Tobias (1943).

Among the most sensitive instruments are those employing the Cartesian diver principle (Holter (1943), Linderstrom-Lang (1943), Anfinson and Claff (1947)). Another respirometer of comparable sensitivity has been described by Gregg (1947); it appears to be easier to operate than the Cartesian diver apparatus and permits measurements to 0.001 µl. Whenever possible it is advisable to work with the Warburg respirometer of the usual size, but when very small pieces of tissue or single small organisms are to be studied measurements with the ultramicrorespirometers are indicated.

The Fenn microrespirometer (Fenn, 1927) is intermediate in its sensitivity between the Warburg apparatus and the respirometers just referred to. It consists of a control flask and an experimental flask connected by a graduated capillary containing a drop of kerosene plus dye. As the tissue respires the indicator drop moves in the capillary. If the two flasks are of equal volume the volume through which the drop moves is equal to one half the volume of gas consumed in the flask containing tissue. This apparatus is entirely satisfactory for measurements of small quantities of gas exchange. Perhaps the reasons it has not enjoyed the popularity of the Warburg respirometer are that its high sensitivity is seldom required and that each measurement necessitates the use of two flasks (as does the Barcroft differential respirometer).

Most of these ultramicrorespirometers as well as others not here mentioned have been described in detail in an excellent recent book by Glick (1949) to which the reader is referred.

W. W. Umbreit and R. H. Burris

THE DETERMINATION OF DISSOLVED OXYGEN BY MEANS OF THE DROPPING MERCURY ELECTRODE

As ordinarily referred to, the dropping mercury electrode is but a portion of the elaborate self-recording instrument known as the polarograph. This instrument plots the current-voltage curve of electrolysis of a solution contained in an electrolysis cell, of which the dropping mercury electrode is the cathode and a pool of mercury is the anode (in certain instances the polarity of the cell is reversed). After standardization of the instrument, the current-voltage curve can be interpreted to determine the substances (of a wide variety), and the concentration of each present in the solution. The unique portion of the polarograph is the dropping mercury electrode; the remainder of the instrument consists of the necessary accessories for applying a constantly increasing voltage, for measuring the current, and for recording the current-voltage data as a continuous curve.

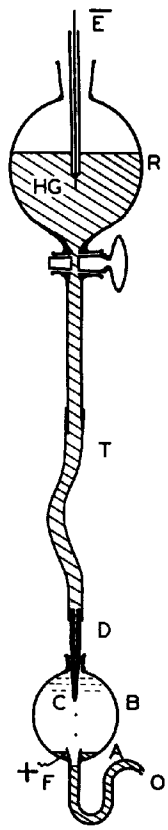

FIG. 35

The dropping mercury electrode.

One type of dropping mercury electrode is represented in Fig. 35. The electrolysis cell, B, contains the solution to be electrolyzed and a relatively large pool of mercury, A, which serves as the anode. The dropping mercury electrode, C, consists of small drops of mercury constantly forming and dropping from the tip of the small-diameter glass capillary tube, D. The reserve of mercury is contained in the separatory funnel, R, and delivered at a constant head (pressure of Hg) through a piece of pure gum rubber tubing, T, to the capillary, D. This arrangement insures a constant drop rate; the rate depends on the particular instrument used and upon the experimental conditions, etc., but the usual rate is one drop every one to one and a half seconds. The mercury pool, A, remains at the same height since the droplets of mercury do not fall into it but instead fall into the column of mercury in the tube below and are discharged through the orifice, O. This type of electrolysis cell has been used in experiments on photosynthesis and respiration by Moore and Duggar (1949). It is preferred to the usual type where the mercury droplets fall into and disturb the anode pool because constancy of distance between cathode tip and anode is assured and the current flow in the galvanometer circuit is very uniform. The dropping mercury electrode assembly is provided with the platinum electrode, E, and the platinum wire, F, sealed into the electrolysis cell, for electrical connections to the circuit of the polarograph.

A diagrammatic representation of a current-voltage curve, such as one might obtain with the polarograph in the electrolysis of a dilute (10^{-4} to 10^{-2} M) solution containing two metallic ions, is shown in Fig. 36. Two abrupt vertical displacements of this curve are shown at A and B, and as the voltage is increased the current increases at a much increased rate for a relatively small change in voltage up to a point where its change approximates the original nearly horizontal portion of the curve. Each region of the vertical displacement of the curve, A and B, represents the voltage where the ions of one of the substances is being completely reduced (or is being discharged) at the dropping mercury electrode (the cathode). The position of A and B referred to the voltage axis gives the "decomposition voltages" E_1 and E_2 of the two substances. Since this curve is reproducible, insofar as the position along the voltage axis where the vertical displacements occur, E_1 and E_2 serve to identify the two particular substances present. During the discharge of an ion, the current becomes directly proportional to the applied voltage as long as the supply of ions to the electrode (in this case the cathode) is not limiting. Finally, however, a voltage is reached where the supply of reducible ions becomes limiting; the current-voltage curve then levels off (it is not exactly horizontal because of the small residual current). As the number of ions of a reducible substance diffusing to the electrode determine the current carried once the decomposition voltage has been reached, it is readily understood that the displacement of the current-voltage curve along the current axis is a measure of the concentration of the particular substance being reduced. Then the vertical distances, C_1 and C_2 respectively represent the concentration of the two substances in the solution, in this case the two metallic ions. Since, at the concentrations employed, ionization is practically complete, C_1 and C_2 represent the molar concentration of the two ions.

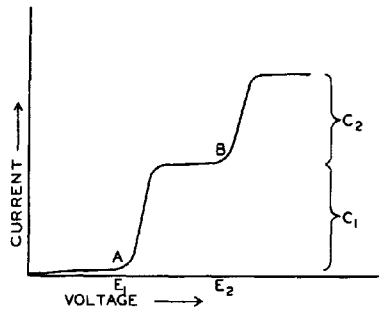

FIG. 36

Current-voltage curve of a solution containing two metallic ions

From the above brief discussion of the principle of the polarograph, it is apparent that the instrument affords a means of rapid quantitative and qualitative analysis (it has many other applications which will not be discussed here, but see Kolthoff and Lingane (1941), and the literature listed in brochures describing the polarograph from apparatus supply companies). The points we wish to emphasize are:

1) Each particular substance which reacts has a decomposition voltage,
2) The difference in the amount of current at the decomposition voltage and at the voltage where the current is limited by the supply of reducible substance is a measure of the concentration of the substance.

On this basis it is possible to effect a simplification of the apparatus required to determine the concentration of one particular substance, in this case dissolved oxygen in biological systems (Petering and Daniels, 1938).

It would appear that if one were analyzing a solution for a single substance, a determination of the difference in current flowing at an applied voltage slightly less than the decomposition voltage of the substance and one in the region of the limiting current would be sufficient to determine the concentration of the substance; furthermore, changes in concentration of the substance could be followed by successive measurement of this "current difference". That the above is true for dissolved oxygen, has been shown by Petering and Daniels (1938). As they point out, since only two voltages (0.1 and 1.0) are necessary for the analysis, the apparatus set-up is very simple, i.e., the dropping mercury electrode assembly (Fig. 35) is connected into a circuit having a galvanometer and either a potentiometer or two large capacity standard cells. These types of the circuit are diagrammed in Fig. 37.

The dropping mercury electrode is easily assembled. A separatory funnel or a leveling bulb serves to contain the supply of chemically pure mercury (the mercury may be reclaimed by cleaning it with dilute (1 to 10) nitric acid, followed by distillation), which is supplied to the glass capillary through a piece of Tygon tubing, pure gum rubber tubing, or

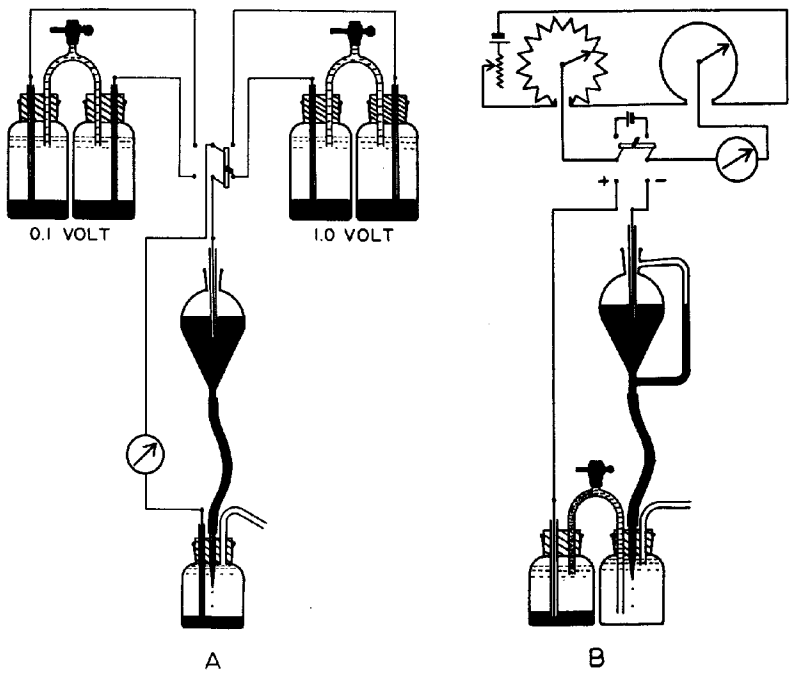

FIG. 37

Dropping mercury electrode arranged for the determination of oxygen.

rubber tubing that has been soaked in hot concentrated alkali to free it of sulfur. The glass capillary which has a fine tip (ca. 0.5 mm. O. D.) with the capillary reduced to such a size that the drop rate is one drop every one to one and one-half seconds with a 30-45 cm. "head" of mercury, may be prepared by drawing down a piece of glass capillary of a broken laboratory thermometer. The electrolysis cell may be an ordinary gas bottle fitted with a rubber stopper bearing the glass capillary, an overflow tube and a sealed in platinum electrode, which is submerged in the pool of mercury at the bottom of the bottle (Fig. 37a), or a cell provided with parallel glass (or quartz) windows may be used. The latter, which is particularly adapted to experiments involving irradiation of the cell contents, is equipped with an overflow tube for the mercury and a constricted neck in which the tapering portion of the glass capillary rests, or is ground in to form a firm joint. If the solution to be placed in the electrolysis cell contains cells or enzymes that are very sensitive to mercury compounds, the anodic pool of mercury may be placed in a separate cell which contains saturated KCl-calomel and is connected to the dropping mercury electrode vessel by means of a saturated KCl-agar bridge (Fig. 37b).

Any available type of potentiometer, a "student type" for example, may be used to supply the two potentials (0.1 and 1.0 volts). It is essential that the potentiometer be frequently standardized during an experiment with a standard cell for slight changes in applied voltage lead to erratic "current differences". As indicated above, the two potentials may be obtained from two standard cells; their preparation is described by Petering and Daniels (1938).

An ordinary type galvanometer with a 10 cm. scale or the standard wall reflecting galvanometer will serve to measure the current; one having a sensitivity of 5×10^{-7} ampere per scale division is sufficient (Petering and Daniels, 1938). We have successfully used an inexpensive galvanometer with lamp and scale (sensitivity, 0.025 microampere; period, 3 seconds). The ground glass scale was replaced with a curved transparent centimeter ruler whose back had been rubbed with fine emery paper to increase the sharpness of the image of the lamp filament. The galvanometer sensitivity may be reduced by use of a suitable shunt.

The dropping mercury electrode is calibrated against known concentrations of oxygen by approximately simultaneously determined current differences and oxygen concentrations; oxygen is determined by the Winkler method.

But before giving the details of the method of calibration, it will not be amiss to point out that the following conditions must be adhered to in order that the calibration may be used to determine the oxygen concentration during an experiment and from one experiment to the next:

1) The solution must be of the same composition.
2) The mercury pressure (i.e., the distance from the tip of the capillary to the surface of the mercury in the reservoir) must be maintained constant.
3) The same capillary must be used.
4) All determinations should be made at the same temperature.

The calibration is as follows: prepare about five liters of nutrient solution, or buffer and add to it the cells or preparation which will be used in the experiments (only a small quantity need be added) or simply pass the liquid through filter paper. This is necessary to eliminate or suppress the "absorption maximum" in the current-voltage curve (c.f., Petering and Daniels, 1938; Kolthoff and Lingane, 1941). Distribute the solution in five liter flasks, each of which is equipped with a rubber stopper bearing a siphon and a gas intake and outlet tube. Saturate the solution in one flask with air at the temperature of calibration, by passing it in through the siphon. The solution is now siphoned out of the flask into the electrolysis cell. Allow the solution to be displaced several times with the tip of the siphon being held near the bottom of the cell. Insert the glass capillary, and proceed to determine the galvanometer deflection at 0.1 and 1.0 volt respectively. Fill two 250 ml. glass stoppered bottles, whose volume is exactly known, with the solution, and analyze for oxygen immediately by the Winkler method (Treadwell and Hall, 1937). Reduce the oxygen content of the solution in one of the remaining flasks by passing in nitrogen (air with its oxygen content reduced by passage through alkaline pyrogallic acid may be used instead) for a few minutes and vigorously shaking the flask. Determine the current difference and the oxygen content as before. Repeat these determinations with successively lower concentrations of oxygen in the remaining lots of solution. The current differences are now plotted against oxygen concentrations to obtain the calibration curve. The points should fall on or very close to a straight line. This calibration curve may now be used to determine the oxygen concentration, or to follow changes in oxygen concentration when the electrolysis cell contains the solution plus the experimental material.

As to the change in oxygen concentration which can be measured by this method, Petering and Daniels (1938) show that when an electrolysis cell containing about 10 ml. is used a total change in oxygen concentration of the order of 5×10^{-9} mole can be determined; this corresponds to 0.112 µl. and to 0.016 p.p.m. of oxygen at room temperature.

This method has been shown to be suited to the determination of the oxygen changes during respiration and photosynthesis of algal cell suspensions, during respiration of yeast, blood cells and animal tissues (Petering and Daniels, 1938). Some specific references of its use, are as follows: photosynthesis (Petering, Duggar and Daniels, 1939; Dutton and Manning, 1941; Moore and Duggar, 1949); oxygen content of soils (Karsten, 1938); respiration of yeast (Anderson and Duggar, 1941) and single oat coleoptiles (DuBuy and Olson, 1940).

J. F. Stauffer

MEASUREMENT OF GROWTH RATES

In general bacterial enzymes are studied with resting cells or cell-free preparations as described in Chapter 10. However, it is desirable at times to follow the metabolism of growing microorganisms. With proliferating cells respiration is no longer linear with time but logarithmic, i.e., a plot of the logarithm of activity per unit time against time will yield a straight line in the ideal case. It cannot be assumed that every measure of activity, oxygen uptake, CO_2 output, methylene blue reducing power, etc., will exhibit a logarithmic increase with time for every organism. The validity of the assumed relationship must be established by concomitant estimation of increasing cell numbers, cell mass, or cell nitrogen. In studying growth processes, cells in their logarithmic growth phase should be used to avoid the complications introduced by the lag phase or phase of negative acceleration.

Burk (1934) reviewed a considerable amount of his experimental work with growing cultures of azotobacter. He demonstrated that in cultures of azotobacter fixing molecular nitrogen the rate of respiration was a measure of growth, and that the growth rate was determined by the rate of nitrogen fixation. Although the relationship was not absolutely quantitative, measurements of oxygen uptake still constituted an excellent measure of growth or nitrogen fixation. He expressed the velocity of growth or nitrogen fixation as the first order velocity constant g (Wilson, 1940, has preferred to substitute k for g to avoid confusion with g as an expression of generation time) where

$$(48)\quad g = k = \frac{2.303\ d \log (a + y)}{dt}$$

$$= \frac{dy}{(a + y)\ dt}$$

a is the initial concentration of azotobacter and y is the increase in t hours. Thus, k is related to the generation time, or number of hours required for doubling the cell concentration, by

$$(49)\quad k = \frac{2.303 \log 2}{\text{generation time}} = \frac{0.695}{\text{generation time}}$$

By plotting the logarithm of the respiration rate for unit intervals against time, k can be evaluated as the slope of the resulting line times 2.303. The k value as a measure of rate is much more valuable than a mere measurement of initial and final numbers or concentrations, for these latter evaluations are subject to unnoticed shifts in rate during the course of the reaction.

The following will serve to illustrate the manner of setting up, observing, and plotting an experiment with a growing culture. To 40 ml. of nitrogen-free medium 4 drops of a 24-hour culture of Azotobacter vinelandii was added, the inoculated medium was shaken vigorously, and 2 ml. was added to each Warburg flask. Since the rate of respiration increases with time it is necessary to start with light suspensions of the organisms; an initial uptake of 20 to 70 µl. oxygen per hour per flask is suitable. The gas mixtures were added to the flasks by the evacuation procedure outlined in Chapter 5. After bringing the flasks to temperature equilibrium at 31°C., the manometers were closed and readings taken at 30-minute intervals thereafter. In Table XIX we have listed the µl. uptake for each 60-minute period as recorded for duplicate flasks; only two gas mixtures of the experiment are shown.

The data from Table XIX are plotted in Fig. 38 with time as abscissa and with oxygen uptake per hour and logarithm of oxygen uptake per hour as ordinates in parts I and II respectively. Plot I indicates increasing rates of respiration by the increasing slopes of the curves with time. Plot II is linear; the straight line through the points is best

TABLE XIX

Hydrogen Inhibition of a Growing Culture of Azotobacter vinelandii

Gas Mixture	Flask	0-60 min.		30-90 min.		60-120 min.		90-150 min.		120-180 min.	
		µl. O_2 uptake	log µl. O_2 uptake	µl. O_2 uptake	log µl. O_2 uptake	µl. O_2 uptake	log µl. O_2 uptake	µl. O_2 uptake	log µl. O_2 uptake	µl. O_2 uptake	log µl. O_2 uptake
60 H_2, 20 N_2, 20 O_2.	1	63.8	1.805	68.8	1.838	70.5	1.848	74.2	1.871	81.7	1.913
	2	63.0	1.800	68.2	1.834	72.5	1.860	77.7	1.891	79.2	1.899
60 He, 20 N_2, 20 O_2.	3	64.4	1.809	70.5	1.848	77.0	1.887	88.9	1.949	97.2	1.988
	4	63.2	1.801	69.8	1.844	77.8	1.891	86.8	1.938	94.0	1.973
		150-210 min.		180-240 min.		210-270 min.		240-300 min.			
	1	88.5	1.947	94.8	1.977	100.7	2.002	106.9	2.029		
	2	85.6	1.933	96.0	1.982	103.5	2.015	110.2	2.043		
	3	109.3	2.039	124.5	2.096	137.5	2.138	153.2	2.185		
	4	105.6	2.024	123.0	2.090	139.6	2.145	155.8	2.192		

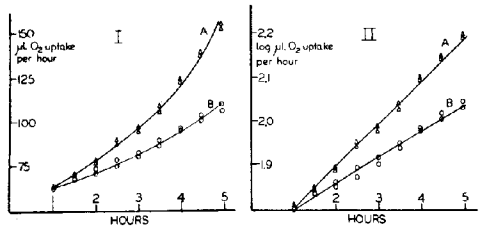

FIG. 38

Rate and log rate plots of oxygen uptake by a growing culture of Azotobacter vinelandii.

calculated by the method of least squares. The slope of the line times 2.303 gives the k value of the culture; from Fig. 38, II, the k values are 0.219 for A and 0.127 for B. As mentioned, the generation time equals 0.695/k, which gives 3.17 hours for A and 5.47 hours for B.

In the experiment described the nitrogen-free medium limited the growth of organisms other than azotobacter. In experiments lasting for periods greater than 4 to 5 hours and employing media subject to ready contamination, respirometer vessels should be steamed before use to reduce the load of contaminants. When it is necessary to follow activity for a day or more it is essential that the flasks and all material added to them be sterilized. Under such conditions flasks designed to retain a cotton plug (see Fig. 22) must be employed. A venting sidearm with cotton plug retainer may be attached for the addition of solutions and for flushing gases through the system, or a solid sidearm will serve if gases are changed by evacuation. Ordinary flasks may be used with the adapters shown in Fig. 23.

When using cotton plugged flasks, sterilize them with the indicated cotton plug in position and another cotton plug in the top. When pipetting the culture into the flask, discard the top plug and remove and replace the lower plug with sterilized forceps. Do not sterilize the medium (or KOH) in the flask; much more uniform results will be obtained if a bottle of medium is inoculated, shaken and then pipetted aseptically into the respirometer vessels.

DETERMINATION OF THE TYPE OF INHIBITION

In interpreting the mechanism of enzyme action it is frequently very helpful to understand the manner in which specific inhibitors function. The usual basic distinction is that between competitive and non-competitive inhibitors. In the case of competitive inhibition the substrate and inhibitor are competing for enzyme surface, and hence changes in either inhibitor or substrate concentration will affect the degree of inhibition observed. Substrate concentration is without influence in non-competitive inhibition.

The mere observation of the constancy of inhibition at one inhibitor concentration and with varying substrate concentration should not be relied upon to establish the competitive or non-competitive nature of the inhibition. The more reliable method is to apply the equations for testing the type of inhibition that have been described by Lineweaver and Burk (1934). Derivation of the equations in detail has been given by Wilson (1939). Ebersole, Guttentag and Wilson (1944) have discussed competitive, non-competitive and other less common types of inhibition, suggested by Burk. These references should be consulted for adequate derivation of the theory upon which the tests for type of inhibition are based.

The application of the tests is relatively easy. Determine the velocity constants, v (the k value discussed under "Measurement of Growth Rates" is an example of a velocity constant), of the reaction over as wide a range of substrate concentrations as is practical and at two or more concentrations of inhibitor. As pointed out by Ebersole, Guttentag, and Wilson (1944), if the reciprocal of the velocity constant, $1/v$, is plotted against the reciprocal of the concentration of substrate, $1/(S)$, straight lines (calculated by the method of least squares) should result with the following characteristics:

I. In the absence of inhibitor, a straight line results whose slope/intercept equals K_s, the dissociation constant of the enzyme-substrate complex.

II. In strictly competitive inhibition, the intercept remains constant, but the slope is increased by $(1 + (I)/K_i)$, where (I) is concentration of inhibitor, and K_i the dissociation constant of the enzyme-inhibitor complex. The apparent K_s = slope/intercept increased by the same factor, i.e., $(1 + (I)/K_i)$.

III. In strictly non-competitive inhibition, both the slope and intercept are increased by the same quantity, viz., $(1 + (I)/K_i)$ so that K_s remains constant.

Figure 39, from Wilson (1939), shows a plot of the reciprocal of substrate concentration, $1/pN_2$, against the reciprocal of the velocity constant, $1/k$, at varying inhibitor concentrations, H_2 pressures of 0.2, 0.4 and 0.6 atmospheres. The intercepts are common,

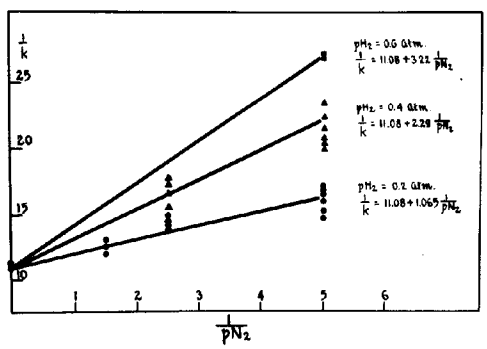

FIG. 39

Hydrogen as a competitive inhibitor of nitrogen fixation by red clover.

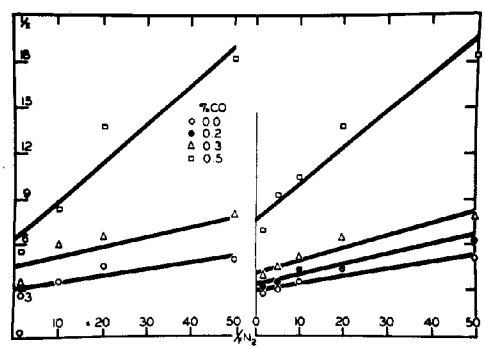

FIG. 40

Carbon monoxide as a non-competitive inhibitor of nitrogen fixation by Azotobacter vinelandii.

but the slopes increase with increasing inhibitor concentrations indicating that H_2 acts as a competitive inhibitor in the symbiotic nitrogen fixation system of red clover. Fig. 40, from Ebersole, Guttentag and Wilson (1944), shows an example of non-competitive inhibition, namely the CO inhibition of non-symbiotic nitrogen fixation by Azotobacter vinelandii. In this case the intercepts and slopes both increase with increasing concentrations of the inhibitor, CO.

If the data do not fit the described conditions the inhibition may be of the "uncompetitive" or "quadratic" type. Consult Ebersole, Guttentag and Wilson (1944) for a description of tests for determining inhibition of such nature.

THE USE OF ISOTOPIC TRACERS IN THE WARBURG APPARATUS

The use of isotopic tracers to follow enzymatic reactions has not been exploited extensively as yet but holds great promise for the elucidation of reaction mechanisms. The extreme sensitivity of the tracer technique permits its application on a micro scale, and the conventional Warburg apparatus is ideally suited for conducting a wide variety of enzymatic and non-enzymatic tracer experiments. This apparatus provides a system in which the gas atmosphere, the temperature and the shaking can be controlled conveniently, gases can be generated or absorbed internally, components can be added to the reaction mixture, and the course of the reaction can be followed manometrically. Furthermore, the all glass apparatus is easy to decontaminate.

It is outside the scope of this book to discuss in any detail the instrumentation, and techniques involved in the use of isotopic tracers. We will give only illustrative suggestions, and for a general discussion of isotopes and working methods the reader is referred to the books by Kamen (1947), Calvin, Heidelberger, Reid, Tolbert and Yankwich (1949), Hevesy (1948), and the Wisconsin Symposium on the Use of Isotopes in Biology and Medicine (1948).

As an example of the application of isotopes to the study of an enzymatic reaction, we will consider an experiment to test the ability of a heterotrophic bacterial species to fix CO_2. Pipette the bacterial suspension (washed cells or growing culture) into the main chamber of a vessel of the type shown in Fig. 30, page 79. Omit KOH from the center well. Place the substrate in the main chamber or in the simple sidearm from which it can be added after the system is closed. Put a mg. of $BaC^{14}O_3$ into the primary sac of the double sidearm and 0.2 ml. of 30% perchloric acid in the secondary sac of the double sidearm. Attach the flask to the manometer and equilibrate the system in the bath. Close the manometer and dump the acid into the $BaC^{14}O_3$ to generate labeled CO_2. Read the manometer when equilibrium is again attained and read at intervals during the run. Control flasks with KOH in the center well and without $C^{14}O_2$ will furnish data on the uptake of O_2 by the cells. At the end of the run, open the flask containing $C^{14}O_2$ in a well ventilated hood so that there will be no chance of breathing the radioactive gas. Acidify the contents of the flask and bubble normal tank CO_2 through the liquid to remove residual $C^{14}O_2$ (or add $NaHCO_3$ to the acidified solution and heat to sweep out CO_2). Remove the contents of the flask with a pipette with rubber bulb or safety pipetter attached (do not pipette by mouth). Separate cells or soluble components from the reaction mixture and measure their radioactivity directly as a dried film, or for more precise measurements convert the carbon to $BaCO_3$ for measurement. As radiocarbon is added only in the form of CO_2, and as all residual CO_2 is removed, it is obvious that the presence of C^{14} in the cells or any compounds in the medium is evidence of CO_2 fixation (non-enzymatic exchange reactions can be ruled out by tests with heated controls).

In an experiment such as the one described, it is often as satisfactory and more convenient to add $NaHC^{14}O_3$ to the reaction mixture rather than to generate $C^{14}O_2$. When bicarbonate is added, flasks with double sac sidearms are not necessary. It is easier to pipette given quantities of $NaHCO_3$ into each flask than to weigh $BaCO_3$ for each or to add a slurry of $BaCO_3$. The $BaC^{14}O_3$ is converted easily to $NaHCO_3$ by placing a slight excess of CO_2-free NaOH in the center well of a Warburg flask to absorb CO_2 generated by adding acid from a sidearm to $BaCO_3$ in the main chamber. The manometer will indicate when CO_2 absorption is complete. The solution of sodium carbonate and bicarbonate can be recovered from the flask, titrated to the desired pH and diluted to an appropriate concentration.

The metabolism of many isotopically labeled compounds other than CO_2 can be followed conveniently in a microrespirometer. For example, in the oxidation of carboxyl or methyl labeled acetate by microorganisms the radioactivity of the CO_2 collected during the reaction can be checked to determine whether it originates solely from the carboxyl group or from both the carboxyl and methyl groups. Products arising by condensation of acetate or acetate fragments will reveal their origin by their radioactivity. By using carboxyl and methyl labeled acetate in turn, the carbon atom involved is defined. If the bacteria are capable of oxidative assimilation of acetate, the label from the acetate will appear in the bacterial cells or in the layer of polysaccharide on the cells. Reactions such as these often can be followed with the 2 or 3 ml. of reactants normally used in a Warburg flask. The recovery of some products may be feasible only when the contents of several flasks are pooled or when larger reaction vessels are employed. The carrier technique, involving addition and reisolation of a substance, will aid in recovering a product present in small amounts.

Isotopic carbon is useful in establishing the influence of added substrates upon the metabolism of endogenous substrates. It is difficult to find whether in the presence of added substrates the oxidation of the endogenous materials is completely suppressed, partially suppressed or unaffected. To determine the effect of an added substrate on the endogenous respiration of bacteria the following method may be employed: Grow plants, e.g. bean plants, for a few weeks, and then expose them for a short period to $C^{14}O_2$ in a closed glass system in the light (Burris, Wilson and Stutz, 1949). By photosynthesis they will fix the $C^{14}O_2$ and then convert it to a variety of compounds. Harvest the plants, grind them, and incorporate the ground material into an agar medium. Distribute the medium in Roux bottles, sterilize it, cool and inoculate the slants with bacteria. The bacteria harvested from such cultures will contain radioactive carbon in all or most of their cellular constituents. Allow these cells to respire in the Warburg respirometer with $Ba(OH)_2$ or CO_2-free NaOH in the center well. Recover the alkali at the end of the run, filter off or centrifuge down the $BaCO_3$ (precipitate $BaCO_3$ from the NaOH with $BaCl_2$) and analyze its radioactivity. If in the presence of substrate no radioactivity appears in the $BaCO_3$ collected, the endogenous respiration has been suppressed completely. If the total radioactivity captured in a given time is the same in the presence and absence of added substrate, the added substrate has not influenced the endogenous respiration. Intermediate activities indicate partial suppression of endogenous respiration, and quantitative determinations will show the extent of this influence.

R. H. Burris

A VOLUMETRIC APPARATUS FOR STUDIES OF TISSUE METABOLISM

The volumetric principle in respirometry can be described as follows: The gases are kept under constant temperature and pressure, and changes in volume are read directly.

This has considerable advantages over the manometric principle. By allowing a direct reading of the volume changes the theory of the volumetric respirometers is very simple. No vessel constants have to be determined or calculated. The only part to be calibrated is the volumetric device. Vessels of different volume can be applied without the necessity of recalibrating the apparatus. An essential feature of a volumetric respirometer is a manometer, which here serves to indicate the constant pressure. The manometer should be balanced against a compensating vessel containing a few drops of water. Thereby the apparatus is made insensitive to changes in barometric pressure and humidity during the experiment, and also less sensitive to changes in the environmental temperature than an uncompensated system, so that a less precise thermoregulation is needed. It will generally suffice to keep the water bath within 0.1° Centigrade.

The sensitivity of the manometer can be varied within wide limits. It is more sensitive the finer the bore is, the lighter the manometer fluid, the smaller the respiration chamber, and the larger the compensating vessel.

The volumetric device of the respirometer serves to restore the constancy of the pressure before each reading and shows the magnitude of the change in volume, which has

taken place. It should have a reading accuracy corresponding to the sensitivity of the manometer.

The volume changes are corrected for standard temperature and pressure in the usual way.

The first apparatus including the features mentioned, was described by Winterstein in 1912, 1913. He used as a volumetric device a finely graduated capillary burette. A similar device was used by Dixon (1943). Scholander (1942a, 1949) has improved the design of volumetric respirometers, and introduced his microburette (Scholander, 1946b) for replacement of the oxygen consumed. It has a micrometer caliper unit attached to a mercury chamber, which is connected with an oxygen delivery chamber. The plunger of the micrometer regulates the flow of mercury into the oxygen chamber, from which a corresponding volume of oxygen is transferred to the respiration vessel, until the manometer is balanced again. The volumes of mercury (oxygen) delivered can be read on the micrometer with great accuracy.

By substituting the plungers of the regular micrometers with drill rods of smaller dimensions, Scholander was able to obtain a high degree of accuracy in the delivery of small quantities.

Scholander (1949) has recently designed volumetric microrespirometers in plastic (plexiglass), a material also used by Robbie and Leinfelder (1945) in a constant flow manometric microrespirometer. Scholander uses syringes for oxygen delivery and as measuring devices, either the commercial all-glass type, or a plexiglass microsyringe, where a dial indicator (Gilmont, 1948) is applied to read the position of the plunger.

I was impressed by the possibilities of plastics as material for construction of microrespirometers, and in cooperation with Mr. O. Hebel, I have designed an apparatus for studies of cell respiration and tissue metabolism (Wennesland and Hebel, 1949).

It is based upon the general volumetric principles as outlined above, with a respiration chamber connected to a compensated, very sensitive manometer. The volume changes in the gases of the respiration chamber are counteracted by moving a stainless steel rod in the oxygen delivery chamber, until the manometer is in balance. The position of the plunger can be read with a dial indicator, or with a simplified micrometer device, and gives the volume changes in the gas phase, when the dimensions of the plunger are known.

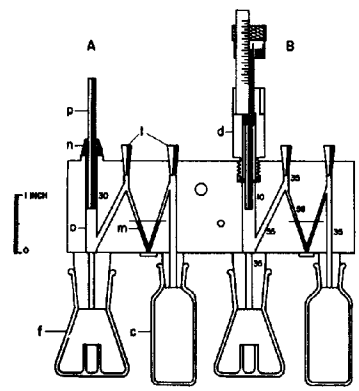

FIG. 40-1

One manometer block with two respirometer units. A has the oxygen delivery chamber with plunger for dial indicator reading. B has the micrometer device. The numbers show the drill dimensions used for the various channels.

DESCRIPTION OF APPARATUS

The apparatus (Fig. 40-1) consists of the following parts:

1. Respiration chamber.
2. Compensating vessel.
3. Plexiglass manometer block, which also contains the oxygen delivery chamber.
4. Delivery and measuring device for the oxygen.
5. Mounting and shaking device.

The apparatus can be made by any workshop, which has a drill press, a lathe and preferably for making straight edges on the manometer block, a milling machine.

1. The conventional Warburg flasks (Fig. 40-1,f) with one sidearm and center well may be used.

2. For compensating vessels small bottles (Fig. 40-1,c) with standard size ground glass stoppers (e.g. A. H. Thomas Cat. No. 2232) of approximately the same volume as the

Warburg flasks may be employed. Larger volume bottles will increase the sensitivity of the manometers.

3. The plexiglass manometer block adapted for two respirometers is shown in Fig. 40-1. This arrangement was found practical for attaching the block to the shaking device. One respirometer with plunger (p) for dial indicator reading is shown in Fig. 40-1 A. The micrometer device (d) is shown in Fig. 40-1 B. The position of the manometer (m), the oxygen delivery chamber (o), and the connecting channels is shown from the figure. The numbers to the right of the bores show the drill numbers used. A jig should be used, when the manometer and the oblique channel, which connects the manometer with the oxygen delivery chamber (and the respiration chamber), is drilled. All drillings must be done slowly, only a short piece at a time. If the drill heats too much, the plexiglass will melt, and the work is spoiled. Water or kerosene can be applied for cooling off the drill.

The manometer bores were drilled from below, and the lower opening later sealed with a little plate of plexiglass. The manometer openings were drilled from above, and were molded to fit the small tapered plugs of plexiglass (t), which are used to close them. The molding was done with a tapered brass rod put on a file handle. Its tapered end was of exactly the same size as the plugs. It was heated gently and pressed into the manometer openings, cooled under the water faucet and retracted when it loosened (Scholander, 1949).

If one choses the dial indicator system for reading the plunger position, the oxygen chamber needs a neck piece with a small collar of tygon tubing (Fig. 40-1 A n), to make an airtight fit. The neck piece was machined from a plexiglass rod and cemented to the upper edge of the manometer block, where the oxygen delivery chamber is to be located. After the cement had dried, the oxygen chamber (o) was drilled from the top of the neck piece to the bottom of the manometer block. The collar was made from tygon tubing 3/16 inch O.D., 1/8 inch I.D., which was rotated while heated gently over a flame until it became soft. It was taken out and pulled cautiously to a slight constriction. When the latter was cut in the middle, it provided two sleeves, slightly conical. They were put on to a brass rod and machined to get straight edges.

If one choses the micrometer device (Fig. 40-1 B), the upper part of the oxygen delivery chamber has to be modified to receive the micrometer barrel screw, which is of 3/8 inch diameter and has 24 threads to an inch.

The upper 3/8 inch of the oxygen chamber was drilled with a drill 21/64 inch, and threaded with a bottom tap of same dimension as the barrel screw. The bottom of this receptable was made flat with a 21/64 inch drill ground flat, to support the fibre gasket, which here serves to give an airtight fit. The rest of the oxygen chamber was drilled with drill No. 10. By using a larger oxygen delivery chamber the 1/8 inch plunger generally used, can be substituted by other plungers (for example, of 3/16 inch diameter) where the changes in gas volume are greater than ordinarily.

The tapered joints for the Warburg flasks and the compensating bottles are machined from plexiglass rods and drilled through in the lathe. They are cemented to the manometer block after all channels have been finished.

4. For the delivery of oxygen plungers of stainless steel were found to be the best. Tests showed that the commercial rods do not oxidize, and that they are of uniform diameter. They were polished with fine emery cloth. We generally used rods of 1/8 inch diameter, which delivered about 200 μl. gas on 1 inch's length.

If applied for dial indicator reading, a polished piece of 2 inch's length was cut off and both ends machined. It was greased with Nevastane heavy X (Keystone Company, Philadelphia), or another suitable non-oxidizing grease, and put through the neck piece with the tygon collar into the oxygen delivery chamber. It can be moved freely up and down. However, if left for more than one hour, it has a slight tendency to stick.

For reading its position a dial indicator system (Scholander, 1949, Gilmont, 1948) can be used. The dial indicator (Ames Model 282 with one inch travel divided into 1000 parts) was provided with a firmly attached plexiglass sleeve (Fig. 40-2 s), which has a

bore to fit over the plunger. During measurements it rests against the edge of the tygon collar of the neck piece. The spring tension was removed from the feeler rod, and was substituted by a wire ring (r) for finger control.

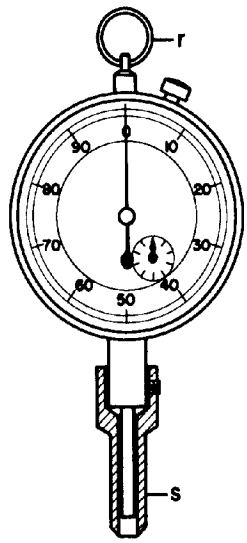

FIG. 40-2

The dial indicator with plexiglass sleeve (s).

The simplified micrometer system (Fig. 40-1 B) has certain advantages over the dial indicator. The screw motion of the plunger allows an easier adjustment of the manometer and a very accurate reading of the plunger position. The oxygen delivery chamber can be provided with plungers of varying diameter, whereby the range of the apparatus can be increased.

The dimensions and various parts of the micrometer device is shown in Fig. 40-3.

All cylindrical parts were made from a plexiglass rod of $\frac{1}{2}$ inch diameter. The micrometer barrel (Fig. 40-3 B) is $1\frac{1}{2}$ inch long. The upper and lower $\frac{1}{2}$ inch parts were machined down to 3/8 inch diameter, the upper to receive the ring which carries the vertical macroscale of the micrometer (Fig. 40-3 A). The lower was provided with the barrel screw, which fastens the micrometer to the oxygen chamber. The barrel has a channel for the plunger, the upper 3/8 inch of which is screw threaded for the plunger screw.

The plungers (F) were made from 1/8 inch stainless steel rods, cut off at a length of 3 inches and both ends machined. The lower 1-1/8 inch serves as a plunger, the rest is screw threaded with a screw die No. 5 with 40 threads to an inch.

The macroscale (A) has a vertical indicator line and 40 divisions to an inch. It was cemented to a ring $\frac{1}{2}$ inch high, $\frac{1}{2}$ inch O.D., 3/8 inch I.D., which was made from a plexiglass rod of $\frac{1}{2}$ inch diameter. Before the 3/8 inch bore was drilled, a small segment was milled off, so that a rectangular plate of plexiglass could be cemented on, to give the macroscale a proper distance from the center (Fig. 40-3, top view of A).

The micrometer screw head (D) was made from an aluminum rod of $\frac{1}{2}$ inch diameter, screwed on to the upper part of the plunger screw and attached with a set screw (E).

The scale of the aluminum screw head which divides each screw revolution into 25 subunits, was engraved with the aid of a dividing head and a milling machine. Also the macroscale was made with the milling machine.

Each division of the macroscale represents one total revolution of the screw. As the screw head divides each revolution into 25, 4 divisions on the macroscale represent 100 of the smallest measuring units, and should be marked correspondingly (Fig. 40-3 A).

The micrometer requires a gasket (C) with a good fit around the plunger. Diamond fibre material was found to be satisfactory.

When the micrometer has been greased, assembled and screwed on to the top of the oxygen delivery chamber (Fig. 40-1 B), the macroscale must be adjusted by the aid of the movable ring, so that it faces the operator, and so that the division lines correspond exactly to the

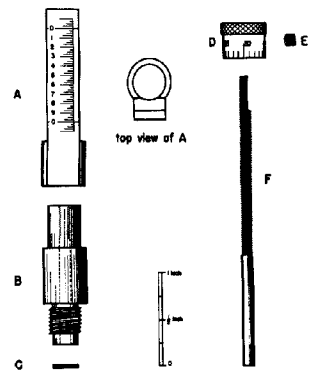

FIG. 40-3

The separate parts of the micrometer device. A, the macroscale with ring attachment (Plexiglass). B, micrometer barrel (Plexiglass). Barrel screw is 3/8 of an inch with 24 threads to an inch. C, diamond fibre gasket. D, micrometer screw head (Aluminum). E, set screw for the head. F, micrometer screw with plunger (Stainless steel). The screw is No. 5 with 40 threads to an inch.

lower edge of the micrometer screw head, when the scale of the latter is in position 0 to the vertical indicator line.

The macroscales and the screw heads are removable. They can be put on to other units of barrels and plungers, when other dimensions are wanted, as long as a screw dimension with 40 threads to an inch is maintained. We make plungers of diameter 3/32 and 3/16 of an inch, and force fit them to the micrometer screw 5 - 40. They will deliver 113 µl. and 452 µl. respectively per 1 inch displacement, divided into 1000 subunits.

At present micrometers are being prepared with 32 threads per inch, so that the commercial plastic rulers can be used for the macroscale, and also for the microscale on the screw heads, by making the latter of plexiglass and cementing a strip of a plastic millimeter scale around its circumference. The strip is heated till it is soft and formed into a circle around a warm metal rod, then cemented to the plexiglass screw head, which has been machined down to receive 25 mm. of the scale, or 50, or 100, depending upon how many subunits one wants for one revolution of the screw (Unpublished).

5. The mounting and shaking device is shown in Fig. 40-4. It was made of a white painted aluminum strip (a) of the same width as the manometer block, 1/8 inch thick and as long as the water bath would allow.

It has a screw and a stud for each manometer block, which fit into the two holes in the middle of the block (Fig. 40-1).

A lock nut (Fig. 40-4 b) can fasten the block steadily to the screw. A double suspension hook (c) was screwed on to each end of the mounting strip. This allows the manometer block to be suspended at two different depths with the manometer openings above (Fig. 40-4 A) and below (B) the water surface.

The shaking rod (d) was made of $\frac{1}{2}$ inch square aluminum and suspended across the edges of the water bath, where it rides on a rig (e) with roller bearings (g). It is driven by a crank (f) and the amplitude and rate of shaking are of the same order as with the conventional Warburg apparatus.

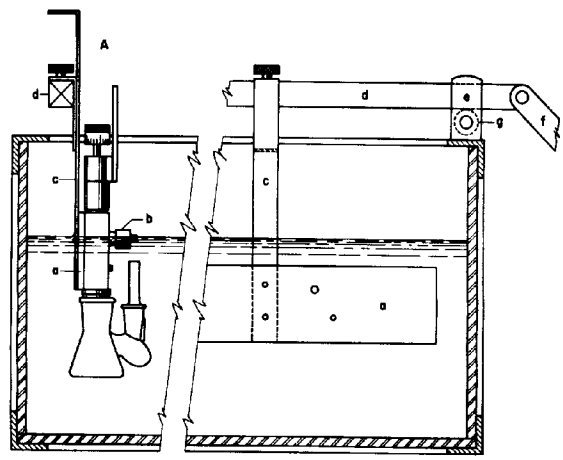

FIG. 40-4

A, side view of mounting device and shaking rod with one respirometer attached. Suspended in the upper position with manometer bores above water surface. B, right end of the same without respirometer. Suspended in the deepest position.

CALIBRATION

The apparatus is calibrated by measuring the diameter of the plungers by means of a conventional micrometer caliper (e.g. from L. S. Starrett Company, Athol, Massachusetts).

The commercial stainless steel rods were perfectly uniform in diameter.

For extreme accuracy the rods should be measured at the temperature of the experiments, or the thermal expansion should be calculated. (The thermal expansion coefficient of stainless steel rods "18-8 type 303" is 17.18×10^{-6} per degree Centigrade.)

The feed of the micrometer screw can be tested against a regular micrometer head (L. S. Starrett Company).

Plexiglass micrometers with no detectable slack of the screw showed that the feed can be made true within 1/1000 of an inch.

If the screw threaded channel in the plexiglass barrel should be worn, it is easy to make a new barrel. There has been no sign of wear in any of our micrometers; however, they have only been in use for about 3 months.

The barrel can be made from stainless steel or monel metal, which will make it more resistant to wear, but heavier.

OPERATION

The operation will be described as used for the determination of the oxygen consumption of respiring material in a medium such as Krebs-Ringer's phosphate solution. The carbon dioxide produced is absorbed in alkali.

After the plunger, or the micrometer device with its gasket thoroughly greased, have been attached to the oxygen delivery chamber, the apparatus is fastened to the mounting device.

The grease is also applied to the tapered joints, and the compensating vessels, containing a few drops of water, are attached.

With a long syringe needle (gauge 24) the manometers are filled with the manometer fluid, water with a little detergent, e.g. liquid potassium soap and some dye, such as Evans blue, up to the level line. The tissue or other respiring material is placed in the Warburg flasks, which contain the medium, and in the center well about 0.2 ml. of 5% KOH plus filter paper.

The flasks are put on to the tapered joints without too much pressure. They can be secured with a rubber sling. However, when the experiment is carried out above room temperature, the thermal expansion of the plexiglass joints (which is a little greater than that of glass), gives a good and solid fit. Thus the compensating vessels will stick to their joints without extra securing devices.

If one wants to run the apparatus using pure oxygen as the gas phase, the oxygen delivery chamber and flask are flushed with oxygen passed into the sidearm of the flask.

After the sidearm has been closed, the mounting strip with the respirometers attached is fastened to the shaking rod in the upper position (Fig. 40-4 A) with both manometer openings above the water level. It should be shaken 10-15 minutes for equilibration. Then the manometer openings are closed with the greased plugs. The mounting strip is moved down to the deepest position, so that all gas volumes are under water.

The initial position of the plungers is read, either by using the dial indicator, or with the micrometer device.

At regular intervals the manometers are brought back to balance and readings are made. The difference between consecutive readings is the oxygen consumption for that interval, uncorrected for standard temperature and pressure.

I have found it advisable to write the reading of the macroscale and the microscale separately with a dash between them, for example: 175/12.

CALCULATION

To illustrate the calculation procedure, I have selected a 1 hour run with rat brain cortex slices in Krebs-Ringer's phosphate solution; readings every 15 minutes. Temperature: 37.5° Centigrade. Barometric pressure at start: 765 mm. Hg.

In the center well about 0.2 ml. 5% KOH plus filter paper. 1 ml. of the medium. 24 mg. rat brain cortex slices were suspended in the medium, using the moist cold box technique (Field, 1948). The flasks were flushed with 100% oxygen through the sidearms.

Time	Readings in micrometer units	Difference, micrometer units
10^{00}	175/12	
10^{15}	275/ 2	90
10^{30}	350/18	91
10^{45}	450/ 4	86
11^{00}	525/15	86

Total difference in 1 hour 353 micrometer units

Micrometer plunger calibrated at 37.5° Centigrade: 0.127 inch diameter. Volume of 1 inch of the plunger (1000 micrometer units) at 37.5° C.: 0.2075 ml.; 1 micrometer unit: 0.2075 µl.

Volume of oxygen consumed in 1 hour: 0.2075 x 353 = 73.25 µl. at 37.5° Centigrade and 765 mm. Hg. (Use the barometric pressure at the start of the experiment. When closing the respirometers with the tapered plugs, the inside pressure is slightly increased; as this amounts only to a fraction of a mm. of Hg., it can be disregarded.)

Corrected for standard temperature and pressure: 64.81 µl. O_2 per 24 mg. wet weight. Using wet weight/dry weight ratio (5.15) this gives:

$$Q_{O_2} = 13.91 \text{ µl. per mg. dry weight per hour.}$$

* * * * * * * * * * *

In an extensive series of measurements of oxygen uptake by slices of rat brain cortex, the apparatus described here has been compared with the conventional Warburg apparatus (Peiss and Wennesland, 1949).

Six aliquots, each about 25 mg. wet weight of rat brain cortex slices were prepared from the brains of each of 8 adult albino rats. Oxygen uptake was measured on 3 aliquots by the Warburg method and the other 3 by the present technique. Thus 48 Warburg runs were made and 47 volumetric (one sample lost). The liquid phase was Krebs-Ringer's phosphate containing 0.011 M glucose and 0.01 M bicarbonate. Gas phase oxygen. Temperature 37.5 ± 0.01° Centigrade. Initial pH: 7.4, final pH: 7.4 - 7.5.

The mean Q_{O_2}'s were 14.12 and 14.08 respectively. The corresponding standard errors were ± 0.169 and ± 0.171. Statistical analysis showed that the two series did not differ significantly either in respect to the means (Student's "t" test) or to the variability (Fisher's "F" test).

R. Wennesland

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Chapter IX

"THUNBERG TECHNIQUES" FOR ESTIMATION OF DEHYDROGENASE ACTIVITY

It is recognized that the uptake of oxygen and the liberation of CO_2 are in reality only the end reactions of a relatively long series of oxidation-reduction reactions. Frequently it is desirable to study these intermediate reactions by means of the "methylene blue" or "Thunberg technique," which is here described.

THE THUNBERG METHOD

In this method tubes as shown in Fig. 41 are employed. These are provided with a sidearm cap arranged to hold materials to be added to the main tube after evacuation. Air is withdrawn through the evacuation outlet when the sidearm cap is turned so that a hole drilled in its standard taper inner joint coincides with the evacuation outlet. The tube may be closed off after evacuation by turning the sidearm.

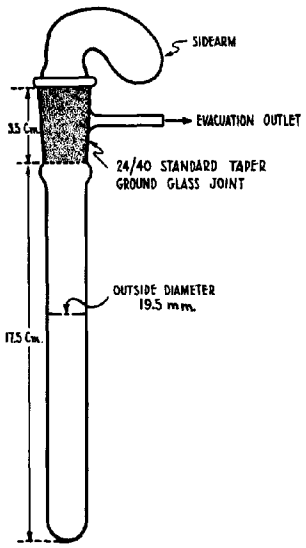

FIG. 41

Thunberg tube suitable for use in a colorimeter.

Distribution and concentrations of reacting components may vary with the application of the method, but the following directions constitute average conditions employed in measurements of methylene blue reduction.

Place 1 ml. 1/10,000 (0.000267 M) methylene blue, 2 ml. M/50 substrate and 2 ml. M/15 phosphate buffer (pH 7.0) in the tube and 1 ml. of bacterial suspension or tissue suspension in the sidearm cap. Put 2 lines of anhydrous lanolin or other grease on the ground joint of the cap at 90° from the hole in the ground joint. Connect the cap and tube with the hole in the cap coinciding with the evacuation outlet on the tube joint. Press the cap firmly in place so the grease from the two lines flows over the entire joint; do not rotate the joint more than a few degrees. By setting the joint in this manner air is not trapped in the grease. Attach the side outlet of the tube to a good water aspirator or a vacuum oil pump, and evacuate for 3 minutes, tapping the tube to release dissolved gas; keep the tube inclined at a low angle to minimize "bumping". A water aspirator is entirely satisfactory for evacuating Thunberg tubes. When evacuation is complete, rotate the cap slowly through an angle of 180°, and then oscillate it through a small arc to set the cap. Put the tube in a constant temperature water bath, and after allowing 10 minutes to reach temperature equilibrium, invert the tube to mix the contents, and follow the methylene blue reduction visually or photometrically. For visual measurement a tube is included which contains all the components of the system being studied (the active tissue is poisoned or heat inactivated) but with the methylene blue at 1/10 normal concentration. This tube represents 90% reduction of the methylene blue, and when the other tubes match the color intensity of this tube the time is recorded as the end point.

The reliability of the results obtained by the Thunberg method depends in part upon the efficiency with which oxygen is removed. Obviously, since leuco-methylene blue is converted into the blue form by oxygen, it will be impossible to measure the real reduction time if some of the dye is being reoxidized by oxygen during the process. It has been found in practice that adequate removal of oxygen is obtained with evacuation by means of an ordinary laboratory water-pump provided the evacuation is continued for at least

three minutes and the tube is tapped vigorously during the evacuation. Fig. 42 from Tam (1939), indicates that methylene blue reduction occurs at approximately the same rate with a three minute evacuation as with evacuation plus an N_2 flush followed by a final evacuation. As Thunberg tubes must hold a vacuum against a full atmosphere of pressure, it is essential that the joints be carefully ground and that they be reasonably long. The 24/40 and the 19/38 standard taper joints are satisfactory.

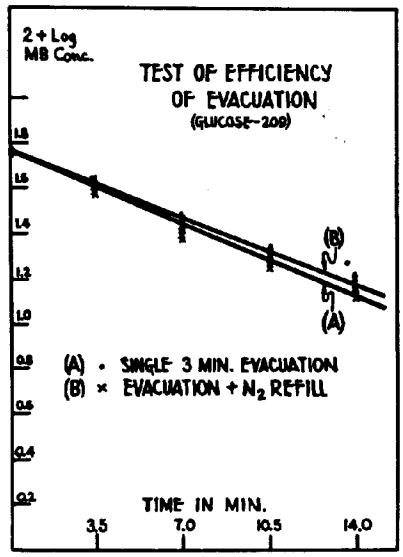

FIG. 42

Effect of evacuation procedure on methylene blue reduction by Rhizobium trifolii 209 on a glucose substrate.

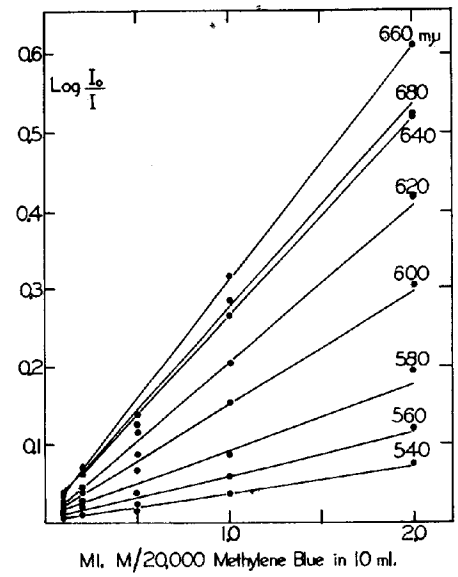

FIG. 43

Absorption of light of different wave lengths by methylene blue.

PHOTOMETRIC ESTIMATION OF METHYLENE BLUE REDUCTION

Methylene blue reduction is not a strictly linear function and much more information on the kinetics of the reaction can be obtained if the reduction is followed photometrically rather than visually. Tam and Wilson (1941) have described such a method using the Evelyn photometer; the tube shown in Fig. 41 is of proper dimensions for this application (such tubes are stocked by the A. H. Thomas Company). With the Evelyn photometer, Tam and Wilson (1941) added concentrations of methylene blue, substrate, buffer and tissue suspension as described in the preceding section. The 660 mμ wave band was used. Fig. 43 shows the light absorption of methylene blue at various wave lengths as determined with the Coleman Universal spectrophotometer; maximum absorption is at 660 mμ and the absorption is a reasonably linear function of methylene blue concentration at this wave length.

To follow methylene blue reduction photometrically a series of tubes are brought to temperature equilibrium, their contents mixed at 15 or 30 second intervals, the tubes wiped dry and initial readings taken immediately after each tube is mixed. The tube is returned to the bath and read every 3 minutes, thus with 15 second intervals 12 tubes can be read every 3 minutes. Suspension concentrations are adjusted so reduction time is from 15 to 30 minutes giving 5 to 10 readings to plot. It is unnecessary to follow the tubes to complete reduction. Upon finishing the experiment a few crystals of sodium hydrosulfite are added to each tube to completely reduce the methylene blue, and the tubes are then read on the photometer, (I_o). The concentration of methylene blue at any time is

proportional to log (I_o/I), where I is the galvanometer reading at any particular time and I_o is the reading after complete reduction. If reduction is linear with time a plot of log (I_o/I) against time will yield a straight line. Tam and Wilson (1941) found that with most substrates the reduction was logarithmic rather than linear with respect to time, hence it was necessary to plot log of methylene blue concentration, i.e., log log (I_o/I), against time. The following data from Tam and Wilson (1941), (Table XX) graphed in Fig. 44, will serve to illustrate the manner in which readings are plotted.

TABLE XX

Effect of pH on Rate of Methylene Blue Reduction by Rhizobium trifolii, Sodium Succinate Substrate.

pH	Log (I_o/I)					2 + Log Log (I_o/I)					Slope of line from Fig. 44a
	0 min.	3	6	9	12	0 min.	3	6	9	12	
4.40	.542	.549	.549	.538	.523	1.734	1.739	1.739	1.731	1.719	.0012
5.25	.569	.523	.482	.453	.417	1.755	1.719	1.683	1.656	1.620	.0112
6.15	.594	.523	.459	.409	.369	1.774	1.719	1.662	1.612	1.567	.0172
6.50	.545	.469	.385	.328	.272	1.736	1.671	1.585	1.516	1.435	.0251
6.95	.561	.456	.367	.310	.254	1.749	1.659	1.565	1.491	1.405	.0287
7.20	.569	.438	.349	.282	.213	1.755	1.641	1.544	1.450	1.328	.0356
7.47	.602	.435	.305	.231	.166	1.780	1.638	1.484	1.364	1.220	.0467
7.83	.594	.429	.301	.197	.128	1.774	1.632	1.479	1.294	1.107	.0556
8.20	.602	.444	.301	.186	.122	1.780	1.647	1.479	1.269	1.086	.0578
8.88	.594	.469	.342	.235	.158	1.774	1.671	1.534	1.371	1.199	.0400

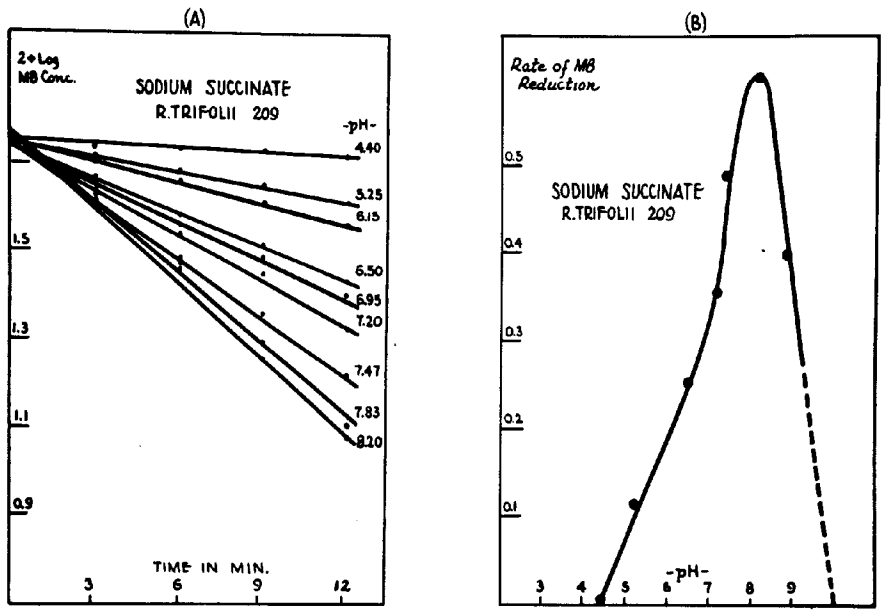

FIG. 44

Methylene blue reduction by Rhizobium trifolii 209 as affected by pH. Sodium succinate substrate.

In the experiment for which data are recorded in Table XX, galvanometer readings (I) were taken at 0, 3, 6, 9, and 12 minutes; end point galvanometer readings (I_o) for each tube were taken after the addition of sodium hydrosulfite. The values for log (I_o/I) are recorded in the table. The logs of these values were then taken. To each number, 2 was added to avoid plotting on a negative scale; this addition does not alter the slopes of the lines. By plotting time against 2 + log log (I_o/I) the straight lines of Fig. 44a are obtained; their slopes can be determined from the graph. In Fig. 44b the slopes (rates of methylene blue reduction) are plotted against pH to give a pH-activity curve for methylene blue reduction by Rhizobium trifolii 209 on a succinate substrate.

It is of interest to point out the wide variety of studies which can be made with a technique of this sort. Tam and Wilson (1941), for example, determined pH optima of several substrates, the temperature relationships which permitted the calculation of energy of activation, the comparative dehydrogenation of a wide variety of substrates, and the effect of a variety of inhibitors.

SIMPLIFIED METHODS

When it is necessary to make many simultaneous estimations of methylene blue reduction time, the number of Thunberg tubes available may restrict the observations. If limited accuracy will suffice, a simplified technique using ordinary test tubes without evacuation may be employed. Such a procedure has been described by Friedeman and Hollander (1942). The following is the modification sometimes used in our laboratory on bacterial suspensions:

To an ordinary test tube add 0.5 ml. of substrate and 0.5 ml. of 1/4000 methylene blue. Add 2 ml. of 2% agar, in 0.5% K_2HPO_4 adjusted to pH 7.0, which has been melted and cooled to 45° C. Add 1 ml. of the cell suspension, mix, and chill in an ice bath until solidified (1 to 2 minutes). Place tubes in a water bath at 37° to 40° C. and determine the time required for reduction. A blue zone (2 to 3 mm.) at the top of the tube results from the diffusion of oxygen from the air and offers a sharp contrast to the reduced portion below.

OTHER RELATIONSHIPS

Methods for measuring methylene blue reduction other than those described but employing electric photometers have been described especially by Ganapathy and Sastri (1938) and by Jongblood (1938). Methylene blue and other dyes sometimes exert a toxic effect on the tissues; Quastel and Wheatley (1931), Yudkin (1933), and Tam and Wilson (1941) report that free phosphates protect against this effect, hence the usual methods employ buffers high in phosphate. As is true of many other cases the method of growing and treating the tissue to be studied may have a marked influence on the rates of dehydrogenation obtained; an example is shown by the work of Wood and Gunsalus (1942).

At times methylene blue (or other dye system of proper potential) may be used to "by pass" a normal system in the cell; for example, its use in restoring the respiration of red blood cells poisoned with cyanide (Barron and Harrop, 1928) has become classic. In some reconstructed enzyme systems methylene blue may be used as an actual carrier of the hydrogen.

By establishing anaerobic conditions with an atmosphere of hydrogen in Warburg vessels Wilson, Lee and Wilson (1942) and Wilson, Burris and Coffee (1943) followed hydrogen uptake by hydrogenase preparations when methylene blue was supplied as hydrogen acceptor. It was necessary to use high concentrations of methylene blue, as the reduction of 1 ml. of a M/1,000 solution requires only 22.4 µl. of hydrogen.

R. H. Burris

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Chapter X

METHODS FOR PREPARATION AND STUDY OF TISSUES

INTRODUCTION

The manometric methods described are accurate and capable of measuring certain reactions with speed and precision. When applied to living tissues or preparations therefrom they will establish the rate and the course of many important processes. Since these reactions are carried out by enzymes and are influenced by an array of physical and physiological conditions, the material employed in the manometric estimations must contain the components of the reaction to be studied.

That some of the multitude of interrelated chemical reactions which occur in vivo could be obtained in vitro has permitted the advances which have occurred in our knowledge of respiration and metabolism. A problem which faces every investigator is the development and study of the means by which reactions occurring in intact tissues may be separated and studied. The techniques applicable to one type of tissue are not necessarily suitable for another. It is, in fact, the knowledge of the physiology of the tissue being studied which permits the selection of a technique of preparation which will be suitable for the tissue employed and the reaction or process one wishes to measure. We wish to emphasize that the techniques described below are not mutually exclusive nor are they all equally applicable. The question of which method to apply (if any) of the techniques listed is the responsibility of the individual investigator just as is the responsibility for the interpretation of the results. The techniques described below are useful but modifications of these or even the development of entirely new ones may be necessary to approach specific problems.

The subjects considered in this Chapter are divided into three sections dealing with Animal, Plant and Microbial Tissues.

METHODS OF PREPARING ANIMAL TISSUES

TISSUE SLICE TECHNIQUE - INTRODUCTION

The tissue slice technique was developed in most of its details by Warburg and his co-workers in their studies of tumor metabolism (Warburg, 1926). By the use of tissue slices, the more complicated and uncontrollable aspects of whole organ or organism metabolism are minimized on the one hand, and the less certain effects of mincing, homogenizing or extracting are excluded on the other hand. In a word, the tissue slice is thought to represent organized surviving tissue, the metabolism of which qualitatively, if not quantitatively, reflects that of the original tissue. Further, the tissue slice technique allows for controlled variations in the suspending medium in addition to chemical analysis of the latter for changes in metabolite content. It should be pointed out that most investigators assume that simple substrates are freely diffusible into slices. That this may not be true in all instances, is seen in the case of liver slices which have a limited permeability for sodium glutamate (Cohen and Hayano, 1946). A critical analysis of the tissue slice method in manometric experiments has been published by Laser (1942).

PREPARATION OF SLICES OF ANIMAL TISSUES

It is usually possible with practice to slice most animal organs free hand with either a straight edge or a safety razor. However, where the size of the organ is very small, as for example a small tumor nodule, a mouse kidney, etc., or where the organ lacks consistency, such as brain, the free hand method of slicing even by the expert leaves much to be desired. A simple and most effective improvement in the technique of tissue slicing applicable to all organs and tissues is that introduced by Deutsch (1936). The principle of this technique is as follows:

The piece of tissue to be sliced is held firmly between two pieces of frosted glass and the tissue sliced by means of a razor blade, the latter being guided by the top frosted glass. In practice it is soon appreciated that the pressure necessary to keep the tissue fixed while slicing varies from tissue to tissue, and further that the thickness of the slice can be estimated by its translucency through the frosted glass.

In the writer's hands the method of Deutsch has proved to be applicable to a wide variety of tissues. The following equipment and procedure has proved most satisfactory.

Equipment:

1) A piece of frosted glass, approximately 5 cm. square, mounted by means of paraffin on a heavy base, 5-6 cm. in height (an inverted cold cream jar has been found very satisfactory).
2) Frosted microscope slides; these are readily prepared from ordinary glass slides by rubbing with emery powder.
3) Razor blades. The three-holed variety broken in half lengthwise are satisfactory.
4) Razor blade holder. This is conveniently made from a piece of brass approximately 10 x 8 x 2 mm. with a hole drilled through one end holding a brass bolt and nut. The nut should have a diameter of about 8 mm. so as to provide a good purchase on the razor blade. The broken blade is placed so that the bolt fits into one of the end holes. The blade is fixed by tightening the bolt with a screw-driver.

Procedure:

The procedure will vary somewhat from tissue to tissue. However, in general one proceeds as follows:

A piece of hard filter paper, approximately 2 cm. square, is placed over one corner of the frosted glass and moistened with saline. The piece of tissue to be sliced (usually about 1 cm. in diameter) is placed on the filter paper. The frosted slide is then dipped in saline and applied to the top of the piece of tissue with gentle pressure and held in place with one hand. The razor blade is then mositened with saline and by means of the holder the blade is closely applied to the under surface of the frosted microscope slide. With experience it will be found possible to adjust the pressure on the tissue with the one hand and effectively slice with the other. The thickness and uniformity of the slice can be readily appreciated by the appearance of the slice through the microscope slide (By cutting across the corner of the frosted-glass base the operator's hand, and the razor blade and holder, will be free of obstruction). The slice is then transferred to a petri dish containing a suitable saline mixture. The slices should be handled by means of a pair of fine but blunt-ended forceps. When transferring the slices to the Warburg flasks, the slices are gently dried by touching with a piece of hard filter paper.

The use of specially designed devices for cutting tissue slices has occupied the attention of many investigators in this field. Very few of these devices have proved to be practical. However, the Stadie-Riggs microtome has had an enthusiastic reception by some workers (Fig. 45). Details of construction and application are given in the paper by these investigators (Stadie and Riggs, 1944). The microtome and blades are obtainable from the Arthur H. Thomas Company, Philadelphia. An excellent review and discussion of the preparation of tissue slices has recently been published by Field (1948).

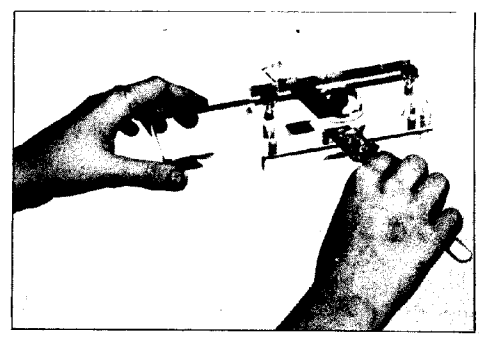

FIG. 45

Stadie-Riggs tissue slicer.

ESTIMATION OF THE THICKNESS OF TISSUE SLICES

Since the rate of diffusion of gases and metabolites will be determined in part by the thickness of the tissue slices, it is essential that they be of uniform thickness within certain limits. These limits are determined by the diffusion constants of the reacting substances, the rate of metabolism of the tissue in question, etc. As derived by Warburg (1930), the limiting thickness in cm., d', for O_2 consumption of slices of a given tissue can be calculated from the equation

$$d' = \sqrt{8\, C_o\, \frac{D}{A}}$$

where D = the diffusion constant for O_2 in ml. (N.T.P.). According to Krogh, the rate of diffusion of O_2 at 38° through a tissue of 1 cm.^{2} cross section is 1.4×10^{-5} ml. per min.

A = the rate of respiration $\left(\frac{\text{ml. } O_2 \text{ uptake}}{\text{ml. tissue x min.}}\right)$

C_o = the O_2 concentration outside the slice (in atmospheres).

Taking 5×10^{-2} as the value for A for liver slices, 1.4×10^{-5} for D, and 1.0 and 0.2 for C_o for pure O_2 and air respectively, d' is calculated to be 4.7×10^{-2} cm. for pure O_2 and 2.1×10^{-2} cm. for air. In other words, if the gas phase is air, liver slices no thicker than 0.2 mm. can be used. Slices of this thickness are not only difficult to prepare but also are very fragile and consequently very difficult to work with. On the other hani, slices 0.3 mm. in thickness can be cut with little difficulty and can be handled and shaken without danger of damage. However, even with slices of this thickness the gas phase must be pure O_2. Under these conditions the O_2 tension at the center of the slice will be about 0.6 atmospheres. Similar calculations have been carried out for CO_2 by Warburg (1930).

In practice it is quite easy to estimate the thickness of a given slice by its translucency and by the manner in which it curls up on itself when held up by a pair of fine forceps. However, in order to establish the correct thickness in terms of the above visual criteria it is best to measure a few selected slices of the different tissues. This is most readily done by placing the slices in a petri dish of Ringer's solution under which is placed a piece of squared millimeter paper. The slices are trimmed to a rectangular shape and their areas measured by counting the squares covered. The volumes of the individual slices are then calculated from their wet weight. The thickness of a given slice is then obtained by dividing the volume by the surface area.

TEMPERATURE OF TISSUE AND MEDIUM

In the author's experience it has been found desirable, and in some instances essential, to keep the tissue and medium at low temperatures while the slices are being cut. This is readily accomplished by keeping the petri dish containing saline and slices on cracked ice, and by filling the cold cream jar with cracked ice and placing it in a petri dish. The latter insures the tissue being kept cold while it is being sliced. The medium to be used for suspending the slices is cooled by storage in the refrigerator up to the time of use. The organs or tissues when removed from the animal are placed on cracked ice directly to chill rapidly and are then placed in small beakers which are surrounded by cracked ice.

DRY WEIGHT OF TISSUE SLICES

Metabolic quotients are usually expressed in terms of mg. dry weight of tissues. As pointed out in Chapter I, this procedure may give rise to erroneous comparisons of metabolic rates of different tissues. Since the metabolic activity is associated more directly with the nitrogen (protein) content of a given tissue, it would seem desirable to include

nitrogen determination when comparing tissues. Experiments in which tissues from an animal on one diet are compared with those from an animal on another diet may show differences which are more apparent than real on the basis of dry weight. This is particularly true in the case of liver the composition of which is so markedly influenced by diet. Thus it is possible to demonstrate an apparent increase in the content of certain enzymes of liver by merely starving the animal. This can be shown to be due in part to the decrease in glycogen with a consequent increase in protein (enzyme) concentration. This precaution of evaluating metabolic quotients, such as Q_{O_2}, on the basis of dry weight in comparative experiments cannot be overemphasized.

Tissue slice dry weights may be obtained in one of two ways:

1. Weighing moist slices on a small torsion balance and then taking a sample to dryness to determine the wet weight/dry weight ratio.
2. Determining dry weights of the tissues in each flask at the end of the incubation without regard to wet weight.

With good slicing technique and care in handling the slices, reliable and consistent results are obtained with either method. The chief objection to the first method is that it is difficult to insure a uniform H_2O and saline content when transferring the slices from the saline to the balance. The question as to whether the one or the other method gives more nearly correct values is not possible to answer. With the first technique it is assumed that the added weight of tissue is maintained and is metabolically active throughout the experimental period. This is certainly not true for all tissues. In the second method, the tissue removed from the experimental flask does not include, usually the fragments broken off during the course of shaking. It is assumed in this instance that these fragments are not contributing to the metabolism of the system. Whether or not this is actually the case it is not possible to say. However, it may in part explain why the Q_{O_2} values obtained with the second method tend to be somewhat higher than those obtained with the first.

For the purpose of determining dry weights of tissue slices, it is convenient to use containers of small weight to allow accurate weighing of a few milligrams. The author uses small flat bottomed vials measuring approximately 10 mm. in length and 8 mm. in diameter, and which weigh between 300-500 mgs. These vials are numbered and their weights recorded. Vials of this size are satisfactory for dry weights of 10-20 mgs.; larger vials are used where the quantity of tissue exceeds this amount. Small watch glasses, crucibles, etc., can also be used. The slices should be dried for about 2 hours at a temperature of 105-110° C.

MINCING OF ANIMAL TISSUES

The purpose in mincing tissues for metabolic study is to reduce them to particles of such size as to permit adequate perfusion in and out of the suspending medium, and also to provide a uniform tissue suspension of relatively high concentration. In comparison with the tissue slice, the mince particles contain a high per cent of damaged cells. The chief use of the tissue mince in recent times has been in the study of muscle metabolism, and in particular, pigeon breast muscle. Several mechanical devices for reducing tissues to a uniform mince have been described and successfully employed in metabolism experiments. A few of these will be discussed.

The feature of the Latapie Mincer (Fig. 46) instrument is that it is possible to control not only the rate of turning of the cutting discs but also the rate at which the tissue is forced against these discs. It is thus possible to obtain minces of different degrees of fineness, by varying the rates of turning of the two cranks, (see Fig. 46). In most metabolic experiments it is desirable to have a mince which can be pipetted after it is suspended in the proper medium. This permits more rapid manipulation and uniformity of tissue content from flask to flask. On the other hand, too fine a mince is usually avoided in order to maintain some degree of integrity of the cells making up the particles. An additional feature of the Latapie is that sterile conditions may be maintained if desired.

The popular Latapie Mincer (obtainable from Arthur H. Thomas Company, Philadelphia) is intended for use with relatively large amounts of tissue, that is, of the order of 25 grams or more. It is thus particularly suitable for a tissue like pigeon heart muscle.

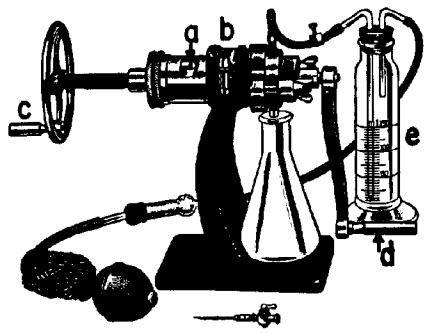

FIG. 46

The Latapie tissue mincing mill. The material to be ground is fed into opening "a" and is gradually forced against the cutting discs at "b" by turning wheel "c". The material is ground by turning the crank "d": the remainder of the apparatus is designed to supply fluid if necessary from "e".

However, where small animal tissues are to be employed the instrument is too large. A smaller model is available for this purpose (Arthur H. Thomas Company, Philadelphia, Pennsylvania).

Seevers and Shideman Mincers:

Other types of small mincing apparatus have also been used. A new type of micro-mincer has been reported by Seevers and Shideman (1941) which appears to be ideally suited for use with small amounts of tissue since it is adaptable to varying quantities of material. Thus the authors report that amounts of tissue from 0.25 to 30 grams can be conveniently minced. The yield of mince from 250 milligrams of tissue is reported as 200 milligrams. In common with the Latapie Mincer principle, the tissue cannot be forced through without being cut since the cutting blades are synchronized to turn as the plunger advances. This feature guarantees a uniform particle size of tissue. A comparison of the respiration of homogenized liver and liver minced by this apparatus has been reported by Potter (1941).

Waring Blendor:

Where relatively large amounts of tissue are available and a fine mince, of the consistency of a homogenate, is desired, the Waring Blendor is recommended.

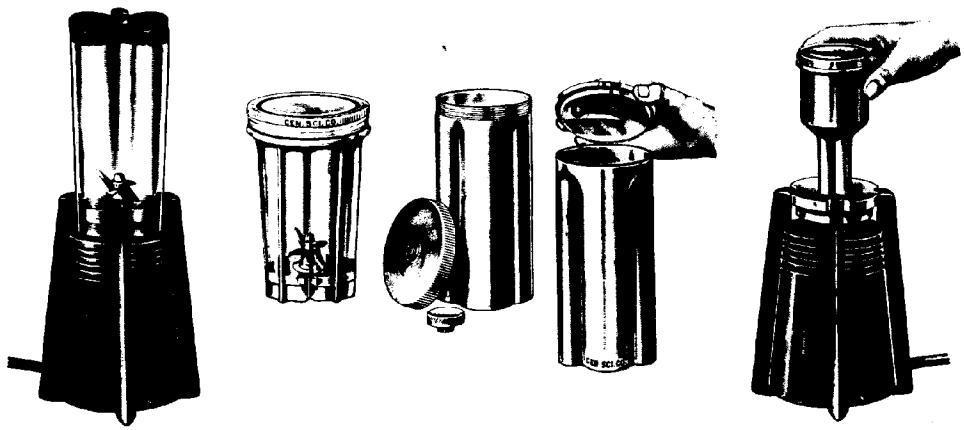

FIG. 47

The Waring Blendor with various containers.

This device has had increasing use in the preparation of homogenates. At present it is possible to obtain a variety of containers varying in content from 25 ml. to 500 ml. (See Fig. 47). Further, stainless steel and aluminum containers which can be sterilized or the contents of which can be handled aseptically are now available.

SPECIAL TISSUE PREPARATIONS

Muscle:

Skeletal muscle does not yield satisfactory tissue slices chiefly for the reason that the muscle cells are relatively large. As a result, slices have a large percentage of damaged cells per slice. Two preparations of skeletal muscle which have been used successfully are fiber bundles described by Richardson, et al. (1930), and young rat diaphragm. The former has been prepared particularly from dog muscle by careful dissection, and the technique was found to yield a uniform preparation of intact fibers. Rat diaphragm is a very convenient muscle preparation since it involves a minimum of preparation. The rats should preferably weigh in the neighborhood of 100 grams. In this weight range, the diaphragm will have a thickness of about 0.3 mm.

Smooth muscle preparations have the disadvantage of contracting to thick pieces of tissue and consequently may not be suitable because of diffusion difficulties. A convenient and little used source of smooth muscle for slicing is bird gizzard. The author has found pigeon gizzard very suitable for slicing since it yields very thin slices which do not contract appreciably. It is further convenient in that a single gizzard will yield enough slices for the most elaborate metabolic experiment. Other convenient smooth muscle sources are:

1. The inner circular layer of the small intestine, which may be dissected free from mucosa and outer layers, and
2. The medial layer of middle-sized arteries from larger animals. The advantage of the latter source of mammalian smooth muscle over that of many others is that one may obtain relatively pure preparations of convenient thickness and amount.

Testis:

The preparation of testis suitable for metabolic experiments is best accomplished by "teasing out" the tissue. The outer capsule is cut with a scissors and the testicular tissue is expressed into saline by manual pressure. The seminiferous tubules are then teased apart by combing the tissue with two pairs of blunt end, curved forceps. This technique insures practically intact tubules.

Retina:

This structure can be used directly in metabolic experiments by merely stripping the sectioned eye ball. Sheep and pig retinae are particularly useful since they represent easily obtainable material. Rat retinae may be used but, of course, a large number of eyes are required to yield a sufficient amount of tissue. Extreme care must be exercised in handling this delicate tissue particularly if anaerobic experiments are to be performed. In the absence of substrate retina may lose a large share of its metabolic activity if kept anaerobic for as little as 2-3 minutes.

P. P. Cohen

WASHED RESIDUE

The finding of Munoz and Leloir (1943) and later of Lehninger (1945) that the fatty acid oxidase activity could be separated from homogenates by centrifugation gave impetus to the development of the washed residue technique (Cohen and McGilvery, 1947, and Cohen and Hayano, 1948) which provides a means of studying certain enzymatic reactions under more easily controlled conditions.

The method now employed in this laboratory is to prepare a 15-30% homogenate using a glass pestle, and with either cold isotonic KCl or a mixture of 80% isotonic KCl and 20% isotonic phosphate buffer of appropriate pH as the suspending medium. The homogenate is filtered through glass wool or gauze to remove gross particles and centrifuged at 2000 g. in the cold for 10 minutes. The supernatant fluid is discarded and the residue is triturated with the original homogenate volume of fresh cold KCl, and again centrifuged at 2000 g. for 10 minutes. This washing may be repeated as many times as desired, although our experience has been that two washings suffice to lower the endogenous respiration to a minimal level. The final residue is made up to a convenient volume with the KCl and added to the experimental flasks.

The increasing body of data being obtained in many laboratories by variants of the above method, indicates that enzyme systems found in the residue will be unstable to freezing, drying, changes in tonicity, and acetone powdering. These systems can not be incubated at 37° in the absence of substrate for even a few minutes without loss of activity toward the usual metabolites (Grisolia and McGilvery, unpublished data). This greatly limits the use of sidearm addition techniques.

On the other hand, the enzyme systems found in the supernatant by this technique are usually relatively stable to solvent and salt fractionation, drying, acetone powdering, etc.

The medium used for studies of washed residue should contain adenosine triphosphate and Mg^{++} ions in concentrations of at least 5×10^{-4} and 2×10^{-3} M, respectively, since these substances are necessary in practically all systems yet investigated. Peculiarly, certain reaction systems require cytochrome c and others do not with the same tissue preparation. If any doubt exists, 1×10^{-5} M cytochrome c should be added. Potassium salts, rather than sodium, should be employed in making up the medium.

The preparation is not cytologically homogeneous and is not adapted for cytochemical studies. On the other hand, the procedures used for the separation into cytologic entities (Schneider, 1948; see Chapter 12) introduces variations in medium, time of manipulation, pH, because of lack of buffering, etc., all of which need careful study with the washed residue preparation itself before special properties are assigned to fractions thereof. In any event, the results obtained with this, or any other broken cell preparation are not quantitatively extrapolatable to in vivo conditions. "Hasty and unfounded applications of the results obtained with damaged cells and the theories built around such results to the chemical processes going on in the living cell have, alas!, crowded the literature and confused thought." (Barron, 1943).

R. W. McGilvery and P. P. Cohen

THE PREPARATION OF CYCLOPHORASE

The cyclophorase complex of enzymes has three outstanding characteristics: (1) all the component oxidases whether flavoproteins or pyridinoproteins are conjugated, i.e., undissociated with respect to their prosthetic groups; (2) all these oxidases can esterify inorganic phosphate coincident with oxidation of the substrate; (3) the complex appears to be associated with particulate elements of a definite size and shape which are probably identical with what the cytologist refers to as mitochondria. In order to prepare the cyclophorase complex essentially undenatured the following conditions must be satisfied: (1) only fresh tissue is to be used for the preparation; (2) all manipulations must be carried out at 0° and with rapidity to minimize both autolytic and thermal inactivation; (3) the final concentration of salt or buffer should be about M/10; and (4) the pH should be maintained between the limits of 6-8. Sucrose can replace sodium or potassium chloride but the complex is less stable in the non-ionic medium. When the salt concentration is reduced to zero or is increased well above M/10 the pyridinoprotein oxidases dissociate. This process of dissociation is irreversible in the sense that adding an excess of coenzyme back to the complex does not restore the original conjugated pyridinoproteins. The apooxidases show catalytic activity when fortified with coenzyme but they are no longer capable of oxidative phosphorylation and their kinetics are profoundly different from those of the corresponding undissociated oxidases of the undenatured cyclophorase complex. The

transformation of the cyclophorase oxidases into their classical counterparts can also be brought about by exposure of the complex to acidity below pH 5 and alkalinity above pH 9, by incubating the complex at 38° in absence of substrate, by treatment at 0° with reagents like arsenite, capryl alcohol and 2,4-dinitrophenol, and finally by prolonged mechanical shearing of the cyclophorase gel in a Waring blendor until the characteristic shape of the particulate elements is lost and a reduction in their size can be observed.

There appears to be an intermediary stage between the cyclophorase and classical state of the pyridinoprotein oxidases. Thus by mechanical shearing of the cyclophorase gel which leads to reduction in size of the particles one can obtain a preparation in which the oxidases are still largely conjugated but in which the property of oxidative phosphorylation is lost. The characteristic of the typical classical state is that the pyridinoprotein oxidases are completely dissociated and lack the property of oxidative phosphorylation. In addition the classical oxidases have been separated from the particles and are in true solution.

The conditions and methods of preparation thus determine to what extent the cyclophorase gel will retain its full catalytic potentiality or will undergo transformation to enzyme artifacts which while retaining catalytic activity have lost the most characteristic properties of the original complex.

The concept of cyclophorase represents a departure from the traditions of enzyme chemistry in that the ending "ase" is now applied to a complex of enzymes rather than to a single enzyme. However, the unit of the cyclophorase system appears to have the same reproducibility of properties as does a single enzyme, and in that sense qualifies for inclusion among the units of enzyme chemistry.

The method of preparation which follows is designed to provide a highly active suspension of the cyclophorase gel in a reasonably stable state and within a relatively short time. The final suspension contains nuclear fragments which contain no activity. However, the procedures which have been found to separate the active mitochondria from inactive nuclear fragments are somewhat more tedious, and they have the added disadvantage that the final preparation is rather unstable.

Preparation of Complex from Rabbit Kidney: A large rabbit (2.5 - 4 kg.) is guillotined or killed by a hard blow with a lead pipe aimed at the nape of the neck and the neck arteries then severed with a sharp knife. The external capsule of the kidneys is removed; the kidney is then split longitudinally into two halves and the medulla, pelvis and connective tissue dissected out. The kidneys are kept in iced water except during handling. The kidneys are then transferred to an ice cold jar of the Waring blendor containing 100 ml. of 0.9 per cent KCl (0°) and 0.24 ml. of N sodium hydroxide. The blendor is allowed to work for 2 minutes during which time the pH of the mixture is determined every few seconds by removing a drop and checking the pH with brom thymol blue. Usually about 0.24 ml. of N alkali must be added during the run to maintain the pH at about 7.2. The homogenate is then centrifuged for 5 minutes in the conical streamlined head of a No. 2 International Equipment refrigerated centrifuge at a speed of 5000 R.P.M. The cloudy supernatant fluid is poured off and discarded whilst the sediment is resuspended in 0.9 per cent KCl to the original volume of fluid. The suspension is broken up by means of a high speed stirrer and the centrifugation procedure repeated. The second and third centrifugations require only three minutes for completion. At the third residue stage the gel is suspended in 0.9 per cent KCl to a volume of about 13 ml. for 2 kidneys and the thick suspension brought to a smooth consistency by agitation with a high speed glass stirrer.

Preparation of the Liver Enzyme: The excised liver is divested of the gall bladder and connective tissue and then blendorized with 300 ml. of 0.9 per cent KCl containing 1.2 ml. of N alkali. During the two minute run approximately the same amount of alkali must be added to maintain the pH constant. The gel at the third residue stage is suspended in the minimum amount of 0.9 per cent KCl necessary to render the suspension pipettable. The volume is usually about 40 ml. of suspension for each liver. The centrifuging times for liver and kidney are approximately the same if the conditions for centrifugation mentioned above can be realized. At lower speeds than 5000 R.P.M. the liver suspensions take somewhat longer to sediment sharply.

D. E. Green

SUSPENDING MEDIA FOR ANIMAL TISSUES

The choice of a suspending medium for a given tissue preparation is to a large measure determined by the nature of the metabolic experiment. In the case of tissue slices it is ordinarily assumed that the purpose of the medium is to provide a solution which because of its ionic composition and its osmotic relation to the cells will maintain the integrity of the latter. Thus, the commonly employed Krebs-Ringer solution (Krebs and Henseleit, 1932) is so constituted as to closely approximate the ionic composition of the mammalian serum, (see below). This medium would seem to provide a physiological extracellular environment and so insure the metabolic integrity of the surviving cells. While this may actually be the case, it would appear from the literature that optimum conditions for a given metabolic reaction with slices often require a medium which is different from the balanced, physiological salt solution. Thus, the synthesis of glycogen from pyruvate by liver slices is most rapid when a medium high in potassium is used (Buchanan, et al., 1942). The medium the latter investigators found to give optimum glycogen formation consisted of (per liter) $CaCl_2$, 5.6 mM.; KCl, 75 mM.; K pyruvate, 60 mM; and $KHCO_3$, 43 mM. As can be seen, this medium can hardly be considered a balanced solution in the usual sense of the word. The respiration of brain tissue is markedly sensitive to variations in ionic composition of the suspending medium. When compared with other tissues such as kidney cortex, testis, liver, yolk sac, and retina, brain stands out as being unusually sensitive to the effects of changes in ionic concentration (Dickens and Greville, 1935).

Many other such instances of the effect of varying ionic concentrations on metabolic reactions with tissue slices have been reported. It is only necessary here to stress that the choice of a nutrient medium for a given metabolic experiment with tissue slices may require considerable experimentation with the composition of the medium before optimum conditions are realized.

The Use of Serum as a Suspending Medium: The use of mammalian serum in place of balanced salt solution would seem to represent the ideal physiological medium for suspending tissue slices. While some differences in Q_{O_2} and R. Q. have been reported (Dickens and Simer, 1931; Canzanelli and Rapport, 1939) the magnitude of the difference is usually not great enough to warrant its routine use. As a matter of fact, in some instances respiration is greater in salt solutions than in serum. Some of the difficulties attending the use of serum are:

1) Its preparation
2) The considerable CO_2 retention, and
3) The uncontrollable variability in its make up from sample to sample.

The latter would seem to be particularly undesirable since it represents an introduction of unknown variables in a system which has as one of its features the control of the tissue's environment.

Suspending Media for Tissue Minces: Since tissue minces contain a higher per cent of broken cells, the choice of a suspending medium would seem to favor one more closely approximating intra- rather than extracellular fluid. However, here again it is not possible to predict what type of medium will be most suitable for any given experiment. In the case of pigeon heart muscle mince, a phosphate-saline medium containing NaCl, KCl, $MgSO_4$ and Na_2HPO_4 was found to give higher metabolic rates than phosphate buffer plus NaCl, or phosphate buffer plus $MgCl_2$ (Krebs and Eggleston, 1940). A study of the effect of electrolytes on the respiration of pigeon breast muscle mince has been reported by Kleinzeller (1940). It was found that the optimal K concentration was 0.0385 M if the medium contained 0.02 M phosphate and 0.0425 M NaCl. When the medium contained 0.02 M phosphate, 0.092 M NaCl and 0.00085 M $MgSO_4$, the optimal K concentration was 0.0034 M. The optimum concentration of Mg was dependent on the concentration of other ions in the medium. Thus in a medium containing "physiological" concentrations of Na, K, and Cl the optimal Mg concentration was 0.0025 M. With a K concentration of 0.0385 M, the optimal Mg concentration is about 0.00125 M.

Phosphate, CO_2-Bicarbonate and other Buffers: From a quantitative standpoint the CO_2-bicarbonate system of the extracellular fluid is the chief buffer system in the body.

In the case of tissue slices, the use of phosphate buffer is somewhat more common since it is easier to use. Dickens and Simer (1931) found no significant difference in the Q_{O_2} or RQ of tissue slices in bicarbonate (or phosphate) Ringer solutions. However, more recently, Laser (1942) has shown that in the absence of CO_2 the maximum activity of tissue slices and reactivity to substrates are maintained for only a short time. Further, it was found that CO_2 stabilized the Q_{O_2} of slices in the presence of substrate for several hours and also insured their ability to oxidize substrates added after an incubation period without substrate. Since it is customary to "gas" phosphate buffered systems with 100% O_2, it should be pointed out that Laser has demonstrated that the rate of O_2 uptake without added substrate declined more rapidly in 100% than in lower O_2 tensions.

The use of buffers other than bicarbonate and phosphate has received relatively little study. In a comparative study of various media buffered with phosphate, bicarbonate and borate, Feinstein and Stare (1940) found that with liver slices there was no essential difference in the O_2 uptake. On the other hand, minced liver showed a higher O_2 uptake with the borate buffer than with phosphate or bicarbonate.

Preparation of Krebs-Ringer-Phosphate and Bicarbonate Solutions:

Solutions:

1)	0.90% NaCl	(0.154 M)	
2)	1.15% KCl	(0.154 M)	
3)	1.22% $CaCl_2$	(0.11 M)	(5 ml. equivalent to 11 ml. 0.1N $AgNO_3$)
4)	2.11% KH_2PO_4	(0.154 M)	
5)	3.82% $MgSO_4 \cdot 7H_2O$	(0.154 M)	
6)	1.30% $NaHCO_3$	(0.154 M)	(gas with CO_2 for 1 hour)
7)	0.1 M phosphate buffer, pH 7.4		(17.8 g. $Na_2HPO_4 \cdot 2H_2O$ + 20 ml. 1N HCl; dilute to 1 L.)

To prepare the Krebs-Ringer solution, the following amounts of the above are mixed:

100 parts of solution	1)	Note: All solutions are isotonic with rat serum, hence can be mixed in any proportion yielding mixtures (of differing composition) which are still isotonic.
4 " " "	2)	
3 " " "	3)	
1 " " "	4)	
1 " " "	5)	

If Krebs-Ringer Bicarbonate is desired, 21 parts of solution 6) are added. The solution is then gassed for 10 minutes with 5% CO_2. For aerobic experiments 5% CO_2-95% O_2 mixture is usually used, and for anaerobic experiments, 5% CO_2-95% N_2. After mixing and gassing the solution, it should be kept in a glass stoppered vessel in the cold, until ready for use. The manometric flasks containing the solution should be attached to the manometers and "gassed" as soon as possible.

If Krebs-Ringer-Phosphate is desired, 12 parts of solution 7) are added in place of the bicarbonate solution. This solution after mixing, is gassed with either O_2, H_2 or air, depending on the gas phase desired.

To simplify the preparation and handling of the above solutions, it has been found convenient to make up solutions 1) to 5) five times the concentrations listed. The more concentrated solutions are stable for months when stored in the cold. For a stock Krebs-Ringer solution, solutions 1) to 5) are made up in the proportions indicated above (taking into account the concentration factor of 5). This will keep in the cold for about one week. To make up the Krebs-Ringer bicarbonate solution 16 ml. of solution 6) are diluted to 100 ml. with the stock Krebs-Ringer solution. To make up the Krebs-Ringer-Phosphate, 10 ml. of solution 7) are diluted to 100 ml. with the stock Krebs-Ringer solution.

P. P. Cohen

METHODS OF PREPARING PLANT TISSUES

HIGHER PLANTS

In general, the higher plant does not possess organs constituted of massive tissues as does an animal. The most actively respiring regions of a plant are those where growth, in the sense of increase in number of cells, is progressing most rapidly, namely the meristem regions of the stem and root, the developing flower and fruit, the germinating seed, and seedling. As a rule every plant cell progressively develops from the meristematic to the mature state. The rate of respiration also decreases as the cell matures; too, as the organ of the plant matures, cells are transformed into dead xylem elements, for example, which further lowers the respiratory rate of the organ. Furthermore, the respiratory rate of the higher plants is considerably less than that of most animal tissues.

Since the course of many experiments on respiration are dictated by the very practical consideration of using enough tissue to be able to measure respiration over a relatively short period of time, the introduction of the manometric methods requiring relatively small quantities of plant tissue has allowed considerably more latitude in the choice of material and type of experiments.

We do not propose to discuss at length the preparation of all the various plant parts for use in the study of the several aspects of respiration. Rather, the discussion will be limited to a few general cases together with indications as to where other accounts can be found.

Storage organs of a number of different types of plants have been used extensively. Potato tubers and carrot roots are good examples. Both have a high food content and contain a relatively high percentage of uniform, parenchymatous cells 80-150 microns in width. In the case of the potato tuber, plugs of tissue are removed from the interior by means of a cork borer having a diameter of 6-12 mm. These plugs are then worked up into slices, 0.4-0.75 mm. thick with a hand microtome. In some cases a series of razor blades separated by washers has been used with considerable success to speed up the slicing. The slices are then washed for 12-24 hours in running tap water, rinsed several times in distilled water, blotted with filter paper and transferred to the solution contained in the manometer vessel. The washing serves to remove organic matter from the cut cells and also eliminates certain inequalities in respiration partially associated with the period succeeding cutting (Steward, 1932). Somewhat the same procedure was followed by Turner (1938) in preparing slices of carrot root. He recommends that the whole organ be sliced and then cylindrical discs be cut from the slices to minimize bruising of the tissue. Marsh and Goddard (1939) used carrot slices 6 mm. in diameter and 0.5 mm. thick suspended in pH 5.9 phosphate buffer in determining the effects of cyanide, azide and carbon monoxide on respiration. Potato tuber slices when kept under favorable conditions (see above) may develop a zone of meristematic cells near their surfaces (Steward, et al., 1940). This possibility is worth considering when using this tissue, and one should not overlook the possibility of a similar situation when using other tissues supposedly composed entirely of mature cells, particularly since meristematic and young cells may have metabolic process patterns which differ significantly from those of mature cells, e.g., substrate utilization (Albaum and Eichel, 1943) and terminal oxidase system (Marsh and Goddard, 1939).

Root tips and segments of roots have also been used. It is relatively easy to grow the plants in liquid culture until the root systems have developed sufficiently to yield the desired quantity of material. Tips of the roots of uniform length and diameter are removed, rinsed in distilled water or in the suspending solution, blotted and transferred to the solution in the vessel of the respirometer. It is necessary to randomize the roots in the different samples to overcome inequalities in respiratory rate. This method works quite well when only the extreme tip of the root is used; presumably segments of roots having root hairs would not work as well owing to variable injury to the very fragile root hairs. Machlis (1944) has described in detail a method for producing barley roots 12-15 cm. long, relatively free of root hairs, and of a uniform diameter slightly under 0.5 mm.

He also describes a method of preparing segments of the roots for use in a study of the effects of certain inhibitors and the four-carbon acids on respiration. Root tips from seedlings are also convenient. The seeds may be sterilized for 10-20 minutes in a 10% solution of freshly prepared and filtered calcium hypochlorite solution, washed thoroughly in sterile water, and, after soaking for several hours in sterile water, germinated at room temperature on moistened, sterile filter paper in petri dishes or large, covered crystallizing dishes. The root tips are excised, blotted lightly and transferred directly to the solution in the vessel of the respirometer (Henderson and Stauffer, 1944). This same technique of seed sterilization and germination was also used in obtaining excised roots tips of tomatoes for culture in a purely synthetic medium.

For further details regarding the culture of excised root tips and isolated plant tissues, see White (1943). Loo (1945, 1946) has described the culture of excised stem tips.

Extracts of plant tissues are easy to prepare and find a wide application in the study of the various respiratory phenomena. In many instances an extract may be prepared by grinding a representative sample of the whole plant, organ, or tissue to a pulp in a mortar. The addition of a small amount of sand may facilitate this operation. For larger samples, a food chopper equipped with a fine-toothed cutter or a blendor (Waring type) may be used to advantage. The more liquid portion of the macerated material can be separated from the debris by pressing it out by hand through muslin or 2-3 layers of fine cheesecloth. The liquid may be further freed of suspended particles by centrifuging or by allowing it to stand 10-12 hours at 2-4° C.; this latter treatment appears to work well in obtaining clear extracts from chlorophyllous tissues. The extract may be used immediately, or mixed with a buffer solution, whichever is most suited to the experiment in question. A variant of the above procedure is to freeze the tissue at -8 to -20° C. overnight, thaw, pass through a food chopper with a fine cutter, and press out the sap through felt or cheese-cloth pads in a hydraulic press. Extracts obtained in this manner appear to be more representative of the tissue as a whole, and, at least at first, more active than when the freezing step is omitted. Bunting and James (1941) in a study of carboxylase and cocarboxylase in barley prepared extracts from seedlings frozen overnight at -12° C. by pressing out the sap through muslin by hand. They also tried pulping the seedlings before pressing out the sap, and found that this treatment gave an extract that was more active than that from the unpulped seedlings. However, the difference in activity of the two disappeared after a time. Sap extracted by hand from barley shoots following overnight freezing at -12° C. and thawing was used by James and Craig (1943) in investigating the ascorbic acid system as an agent in respiration. Albaum and Eichel (1943) prepared extracts of whole embryos of oats as follows: 50 embryos were dissected free of the endosperm, ground in sand in 2 ml. of M/15 phosphate buffer, centrifuged lightly and the supernatant liquid decanted and stored in an ice chest overnight. Berger and Avery (1943) ground oat coleoptiles with twice their weight of water, and filtered or centrifuged the extracts for use in determining dehydrogenase activity by means of the Thunburg technique (see Chapter 9). Albaum and Umbreit (1944) prepared extracts of embryos of germinating oats for use in determining phosphorus transformations (see Chapter 15) by homogenizing the material in a minimum amount of ice cold distilled water with a stainless steel homogenizer (see Chapter 11 for a description of this technique). It would appear that homogenates of plant tissue would be more active, in respect to respiratory activity in general, than extracts from pressed, or ground and pressed tissue because the cell debris on which some of the enzymes may be absorbed, and yet remain active, is not removed. It has been used successfully in the study of transamination in oat seedlings by Albaum and Cohen (1943). However, some one or more enzymes may become inactivated, e.g., succinic dehydrogenase in preparations from spinach leaves (Bonner and Wildman, 1946). Whichever technique, or modification of a technique, one employs in preparing a plant extract in the study of a particular problem, it goes without saying that the effect of each treatment in the preparation of the extract should be examined as to its effect on the activity of the final preparation.

PREPARATION OF ALGAL CELL SUSPENSIONS

There are a number of unicellular green algae which may be grown in the laboratory under standardized conditions as a source of plant material for use in studies of photo-

synthesis and respiration. The suitability of such plants for photosynthetic studies has been outlined by Manning, et al. (1938). In many respects the same considerations hold true for their use in respiratory studies. At present a number of species of Chlorella are maintained in pure culture in several botanical laboratories. Without any intention of implying that Chlorella is the only alga recommended, but because of its wide use at present and because of our experience with it, a general method for its culture is outlined below. With slight modification of conditions such as the composition of nutrient solutions, salt concentration, light, temperature, etc., this method can be used for the production of large numbers of cells of many other algae. In fact the method has been used in culturing a species of Dactylococcus, and with relatively slight changes for growing the brine flagellate Dunaliella salina. The method probably had its inception during the course of the experiments of Warburg (1919) and Warburg and Negelein (1922) on photosynthesis. In its present form it closely follows the description given by Manning, et al. (1938).

It is necessary to have the alga in pure culture. Impure cultures can be diluted, plated out on agar medium in petri dishes, and an uncontaminated colony picked off and propagated (see Bold, 1942, for further details). This is rather time consuming with a long period of waiting until a colony is produced from a single cell. If possible a little of a pure culture should be purchased or obtained from a laboratory where the organism is being maintained. To continue the stock, and as a source of inoculum for the liquid cultures to be described below, the alga can be satisfactorily grown on slants of agar-solidified nutrient medium (see below) in ordinary test tubes. Of course, one must exercise the usual precaution against contaminants; the usual bacteriological methods are applicable. After having built up a sufficient supply of stock cultures, these can be easily maintained by bi-monthly transfers of cells onto fresh slants. The cells multiply readily when exposed to natural or artificial light.

The algal cells to be used in preparing the suspensions are grown in liquid cultures. A loopful of cells from a stock culture is introduced into 100-150 ml. of sterile nutrient solution (see below) contained in a 250 ml. Erlenmeyer flask. The flask is provided with a rubber stopper containing two L-shaped glass tubes. One of the tubes extends almost to the bottom of the flask and serves to introduce air or gas mixtures below the surface of the solution. The bubbles of gas also agitate the solution and render constant shaking unnecessary. The other tube is short and serves as the outlet for the gas mixture. The external ends of both tubes are lightly plugged with cotton. When the assembled culture flasks are to be sterilized by autoclaving, a strip of paper is placed around the rubber stopper where it contacts the neck of the flask, the flasks are placed in a metal tray and a paper cover is fitted over the whole. The tray and contents are removed directly from the autoclave to a cabinet or room that has been recently steamed down and allowed to cool. The flasks are then inoculated with the algal cells. The paper strip is removed and the rubber stopper forced into the neck of the flask. Finally, hot paraffin-bee's wax mixture (4-1) is brushed over the glass-rubber joint to render it gas tight. When this procedure is followed, the flasks are seldom contaminated. The flasks are now placed on a wire rack submerged 4-6 cm. in a shallow glass-bottomed, constant temperature tank. The flasks are connected in series (2-15 flasks) by means of short pieces of rubber tubing. Air or a mixture of CO_2 in air (4-5%, prepared by partial pressure method, stored in a carboy or compressed in a cylinder) is constantly bubbled through the flasks at a rate such that the CO_2 content is uniform in all of the flasks of the series. The cultures are illuminated from below with ordinary incandescent or fluorescent bulbs of sufficient wattage to give the desired light intensity. It is not necessary to shake the flasks constantly; shaking them by hand once or twice daily is sufficient. In practice, cultures of the type described in a 24 x 36 inch tank, illuminated constantly with 6 200-watt unfrosted bulbs at a distance of about 3 feet, produce 100-150 $mm.^3$ of packed wet cells per flask in 5-6 days at 22° C. when aerated with 5% CO_2-air mixture.

The following media are given since we have found them to be satisfactory. For others, see Bold (1942).

Medium for agar-slant stock cultures		Medium for liquid cultures	
$NaNO_3$	0.25 gm.	KNO_3	2.53 gm.
KH_2PO_4	0.25 gm.	KH_2PO_4	2.72 gm.
$MgSO_4 \cdot 7H_2O$	0.25 gm.	$MgSO_4$	2.40 gm.
$CaCl_2 \cdot 1H_2O$	0.25 gm.	$CaCl_2$	0.155 gm.
Cane sugar	4.0 gm.	$FeSO_4$	0.0015 gm.
Bacto-peptone	0.5 gm.	Micro-element solution	1.0 ml.
Micro-element solution	1.0 ml.	Distilled water to make	1.0 L.
Distilled water, to	1.0 L.	Adjust pH to 6.8 with KOH	

The particular micro-element solution used contained in 18 liters of distilled water: 30 ml. of 18 N HNO_3; $LiCl_2$, 0.5 gm.; $CuSO_4 \cdot 5H_2O$, 1.0 gm.; $ZnSO_4 \cdot 6H_2O$, 0.5 gm.; $Ti_2(SO_4)_3$, 1.8 gm.; $MnCl_2 \cdot 4H_2O$, 7.0 gm.; $NiCl_2 \cdot 6H_2O$, 1.0 gm.; $Co(NO_3)_2$, 1.0 gm.; KI, 0.5 gm.; KBr, 0.5 gm.; Na_2SO_4, 0.5 gm.; $K_2Cr_2O_7$, 0.2 gm.; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.5 gm. Its use does not appear to be absolutely necessary when ordinary reagents are employed in making up the media with boron and manganese added (0.5 microgram/ml.).

The contents of a flask may be used directly as an algal cell suspension. When greater concentrations of cells are desired, the cells are concentrated by centrifuging. The usual procedure is to centrifuge out the cells in a graduated 15 ml. centrifuge tube, wash once or twice and resuspend them in whatever medium one wishes to use. For short term experiments no precautions against contamination are necessary. The dry weight, algal nitrogen or phosphorus can be obtained from an aliquot. Suspensions prepared in this manner can be used immediately, or, for certain types of experiments, they may be stored in an ice-chest for several days. The quantity required can readily be measured out with a pipette. Suspensions prepared in such a manner have been used in studying photosynthesis (Manning, et al., (1938); Petering, et al., (1939); Fan, et al., (1943)) and phosphorylation (Emerson, et al., (1944)). Most investigators working on photosynthesis and respiration employ algal cell suspensions prepared in somewhat the same manner (e.g., Emerson and Lewis, 1941; Gaffron, 1940; Pratt, 1943).

J. F. Stauffer

METHODS OF PREPARING MICROBIAL TISSUES

PREPARATION OF BACTERIAL CELLS

Washed bacterial cells have been used widely in studies of respiratory enzymes. Washing is considered to remove most nutrients and thus render the cells "non-proliferating" or "resting". Among the advantages rightly claimed for such preparations are: (a) various bacteria present a wide variety of enzymes for study; (b) the organisms can be grown readily under reproducible conditions; (c) the cells give a uniform suspension that can be pipetted accurately; (d) most washed cells can be stored for some time at refrigerator temperatures without appreciable change in activity; (e) respiration remains linear with time; (f) bacteria are extremely active per unit of tissue; and (g) gas diffusion into the cell is not a limiting factor normally.

The media required for best growth of bacteria vary widely with the organisms concerned. In the case of organisms which produce gums or capsules, it may be necessary to grow the cells on media which are low or lacking in carbohydrate to facilitate centrifugation and to limit the high endogenous respiration characteristic of cells abundant in reserve materials. Aside from such considerations, the cells should be grown on as favorable a medium as possible.

When small quantities of aerobic cells will suffice, they are most conveniently grown on an agar medium in liter Roux bottles. These bottles present a large area which is adequately covered by 75 to 85 ml. of agar. Inoculate the slanted, hardened agar surface with 2 or 3 ml. of a suspension of fresh cells, and distribute the suspension over the agar. After incubation, harvest the cells when they are young and active, even at the expense of a reduction in total crop. To harvest, add 10 ml. of buffer or mixed salt solution (e.g., Ringer's solution, Chapter 16) to each Roux bottle and scrape the organisms from the agar surface with a curved glass rod. Filter through cheese-cloth to remove lumps of agar (by using 2% rather than the usual 1.5% agar less difficulty with breakage of the medium is experienced). Rinse with another 5 or 10 ml. portion of solution. Sediment the cells by centrifugation; resuspend in fresh solution and recentrifuge. Repeat. Place the washed cells in a tube or flask equipped for aeration, i.e., with a rubber stopper holding a glass tube leading to the bottom and an exit tube from the top. Aeration at room temperature before the cells are used serves to exhaust metabolites and reduce the endogenous respiration. Store the cells at temperatures somewhat above freezing.

When anaerobes are to be used, they usually can be grown in stagnant carboys of liquid media and recovered by passage through a Sharples supercentrifuge (Koepsell and Johnson, 1942). Precautions are frequently necessary to maintain reducing conditions during harvest to minimize inactivation of the enzymes.

Large quantities of aerobes quite generally can be produced in aerated liquid culture. Lee and Burris (1943) have described the growth of Azotobacter vinelandii under such conditions. Again the Sharples supercentrifuge provides a rapid means of handling large volumes of a culture medium.

The washed cell suspensions are adjusted by dilution to a point where they will induce a gas exchange of 100 to 200 µl. per hour in a Warburg flask; correspondingly, a dilution such that they will reduce methylene blue in 15 to 30 minutes on a suitable substrate is desirable for dehydrogenase studies. The first dilutions will be empirical, but activity can be correlated with turbidity measurements on a colorimeter, spectrophotometer, visual nephelometer, or a set of $BaSO_4$ tubes of varying turbidity. Subsequent suspensions can be adjusted to a reasonably constant activity by dilution to a standard turbidity. When measuring turbidity photometrically in the usual yellow bacteriological medium, it is customary to employ a red filter (620 or 660 mµ) to minimize the effect of the yellow interfering color. With washed suspensions of bacteria in the absence of such color it is advantageous to use a blue filter, since the instrument is more sensitive to turbidity changes under such conditions. Most stock bacterial suspensions will require about a 10 fold dilution to adjust the turbidity to a range suitable for standardization on a photoelectric colorimeter. For example, a suspension of Rhizobium trifolii is of about the proper concentration when 1 ml. added to 9 ml. of water gives 45% transmittance in an Evelyn colorimeter at 420 mµ., distilled water being used as blank.

Turbidity serves as a means of judging activity for adjusting suspensions, but final cell activity is best expressed as the $Q_{O_2}(N)$, or µl. of oxygen taken up per hour per mg. nitrogen content of the cells. The relative merits of dry weight, total nitrogen, nucleic acid phosphorus, total carbon, and cell numbers as bases for expression of activity, are discussed by Burris and Wilson (1940) and in Chapter 1 of this book. In Chapter 13 directions are given for micro Kjeldahl determinations. Duplicate nitrogen determinations should give a basis for expressing $Q_{O_2}(N)$ values for all observations made with one particular suspension.

CELL-FREE ENZYME PREPARATIONS FROM BACTERIA

The advances in our knowledge of yeast fermentation, which have resulted from studies with cell-free preparations, emphasize the value of such experimental material. In their excellent review of methods that have been employed in producing cell-free bacterial juices, Werkman and Wood (1940) have enumerated the advantages and disadvantages of these preparations. Among the advantages are cited the facts that, the results are not affected by growth and reproduction of cells; cellular permeability is no factor in the measurements; it is possible to isolate and follow single reactions through the use of inhibitors and specific substrates; and it is possible to reconstruct a chain of enzymatic reactions

by the combination of the individual components of the system. The disadvantages include the change in environment suffered by the enzymes detached from the cell, and the destruction or incomplete solution of certain enzymes.

Werkman and Wood (1940) should be consulted for a listing and description in some detail of all the methods which have been employed in liberating bacterial enzymes from the cell. In this discussion we shall confine our attention to methods in current, practical use that require only simple, readily obtainable equipment.

GRINDING WITH POWDERED GLASS

Wiggert, Silverman, Utter and Werkman (1940) describe a simple method for macerating bacterial cells by adding powdered glass and grinding with a mortar and pestle. The bacterial cells are best grown in liquid culture and recovered with a Sharples supercentrifuge, as in most cases several grams of wet cells are required. A subsequent wash and recentrifugation is often advisable to remove excess nutrients. The cells should be harvested while young and active; the loss in total crop by early harvest is largely compensated for in greater unit activity. Obviously the manner in which the bacteria are grown will vary with the organism concerned, and the individual investigator will be best acquainted with the nutrient and environmental requirements of the organism with which he is dealing.

Pyrex glass is powdered by grinding in a ball mill; 24 hours with stone balls but not over 4 hours with steel balls usually suffices for proper powdering. Sift the glass through a 30 mesh screen to remove large particles. When handling the glass powder wear a dust mask.

Mix 3 g. of bacterial paste, 25 g. of powdered glass and 7 ml. of pH 7.0, M/15 phosphate buffer; the consistency will be that of a firm batter. Place 10 g. of this mixture in a chilled 4.5 inch mortar and grind vigorously for 5 minutes. Do not use larger than 10 g. portions; observe the 5 minute grinding period, for additional grinding results in inactivation rather than increased yields. Combine the separately ground portions, and for each 10 g. portion add 2 ml. of phosphate buffer; mix well. Transfer to centrifuge tubes and sediment the glass by 5 to 10 minutes centrifugation at as great speeds as the equipment will allow. With particularly viscous preparations longer centrifugation may be necessary. When working with small quantities of viscous material, sedimentation directly in the Beams centrifuge followed by recentrifugation in the cleaned rotor may save time and give more complete recovery from the glass powder. Remove the supernatant and clarify by 2 to 15 minutes centrifugation in a Beams, Weed and Pickels (1933) "spinning top" air driven centrifuge equipped with a screw top with rubber gasket seal. Such a centrifuge can be constructed at low cost by any competent mechanician or instrument maker. The centrifugal force it will develop will depend largely upon the rotor diameter and the pressure of air used for driving the rotor; hence, it is impossible to state exactly the time necessary for sedimentation. This can be determined empirically by observation of the degree of clarification with varying periods of centrifugation. With a 1.25 in. rotor Wiggert, et al., (1940) report suitable sedimentation in 2 minutes at 175,000 r.p.m.; this speed is obtained with 80-90 pounds air pressure. With a 1.5 in. rotor operating from a 30 pound per square in. air line, we obtain good clarification in 3 to 5 minutes with azotobacter preparations. Other preparations may require considerably longer sedimentation; cooling the juice is sometimes helpful in hastening sedimentation. Remove the supernatant with a pipette; the sediment is tightly packed on the walls of the rotor and is not appreciably dislodged when the rotor is decelerated evenly with the fingers.

In operating the Beams "spinning top" centrifuge the hollow rotor can be completely filled, since sedimentation appears to be as effective as when the rotor is only partially filled. Screw the top of the rotor "finger-tight" against the rubber gasket. Open the air line completely, so full pressure is applied to the stator and lower the rotor into position on the stator. Release the rotor, guiding it as it starts to rotate by having the fingers and thumb encircle it in an "eagle-grip". As the rotor gains speed and passes the "chatter-point", line it up by touching the edge gently with the thumbnail. To stop the rotor, leave the air pressure on at full force, place the fingers around the rotor in an "eagle-grip" and apply pressure gently from all sides. The air stream, upon which the

rotor revolves, serves to cool the fingers, so there is no discomfort in stopping the rotor. Even deceleration of the rotor can be judged by the uniform drop in pitch of the audible note from the centrifuge.

Utter and Werkman (1942) described a modification of the manner of grinding given above. Glass and cells were prepared as usual, but were combined in the proportion of 2 parts of powdered glass to 1 part of bacterial cell paste. Buffer to give the consistency of a thick batter was added. "The grinding was accomplished by passing the bacteria-glass mixture between concentric ground glass cones. The inner cone was rotated by a motor." Such cones can be made from standard taper 24/40 or larger joints or built to specifications by any glassworker (Fig. 48). If a specially built cone is employed it should have a taper of 30°-40°; by bringing the inner cone to a tip of about $\frac{1}{4}$" diameter and constricting the junction between the outer cone and cell paste cylinder, cell loss can be minimized. To make the cones from stock standard taper joints, obtain the inner joint with a drip tip, and seal the tip off flat as close to the ground area as is possible without distorting the joint. Fill the inner joint and its length of tubing with crushed ice, and attach its open end to a motor (geared to about 300 r.p.m.) with a rubber stopper. Place the bacterial paste in the unground end of the standard taper outer joint. Join the outer and inner joints, which are mounted in a horizontal position, start the motor, and by means of a plunger, fashioned from a rubber stopper, slowly force the cell-glass paste between the rotating cones. Catch the extruded material in a chilled dish. Extract the paste with 1.5 ml. of water for each gram of bacteria used. Treat subsequent to this point in the same manner as described for preparations ground with a mortar and pestle.

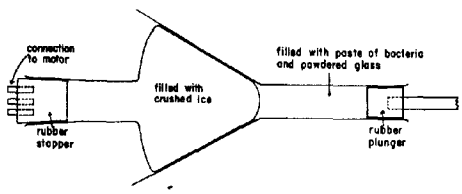

FIG. 48

Glass cones for grinding bacteria. The glassware is Pyrex about 1/8 inch thick.

Juices prepared in this way are essentially free from intact cells. They can be employed directly in respirometer vessels or Thunberg tubes with appropriate additions of buffers and substrates. Any considerable dilution should be avoided, as it may involve a greater than proportionate loss in activity. Wiggert, et al., (1940) discuss the activity of cell-free juices obtained from a variety of bacteria in relation to grinding time, volume of extracting fluid, buffers and substrates employed, addition of viable cells, storage and filtration. Juices from Aerobacter indologenes were completely inactivated by passage through Seitz, Jena glass, or Chamberland filters. With cell-free preparations of Azotobacter vinelandii, Lee, Burris and Wilson (1942) and Lee, Wilson and Wilson (1942) found very little inactivation of the enzymes examined after passage through a Berkefeld N or Mandler 15 filter.

The method of grinding with glass requires simple equipment, is successful with most organisms tested, and comparative studies indicate it has about the same effectiveness as the bacterial mill of Booth and Green (1938) in respect to both speed and completeness of cell disintegration. The Booth-Green mill will not be described here; it can be obtained from Unicam Instruments Ltd., Arbury Road, Cambridge, England.

AUTOLYSIS OF CELLS

One of the simplest means of preparing cell-free enzymes is by autolysis. The ease and the conditions under which different cells will autolyze vary widely. Some cells will autolyze while other cells are growing in the same culture medium, so that the filtered medium will at times carry a usuable concentration of freed intracellular enzymes. Many bacteria, however, are refractory to autolysis, or the autolysis requires such a prolonged time that many enzymes are inactived during the interval. Again the individual must determine the proper conditions for autolysis of the particular cells with which he is concerned.

Stephenson (1928) prepared cell-free lactic, succinic, and formic dehydrogenases from Escherichia coli. In studying the correlation of enzyme activity with number of viable cells present she found that aging suspensions increased in activity on lactate. This observation suggested that autolysis was freeing the enzyme, and optimum conditions for the autolysis were determined. The organisms were harvested, washed, suspended in pH 7.6, M/2 phsophate buffer and incubated at 37° C. for 5 or 6 days in a stoppered bottle. Addition of 1% sodium fluoride prevented putrefactive deterioration, but did not alter the enzyme. The preparation could not reduce molecular oxygen but could reduce methylene blue in the presence of any of the three substrates listed. Filtration through a porcelain filter resulted in complete inactivation. Passage through kieselguhr gave an almost water clear preparation which retained only lactic dehydrogenase activity.

To deal in general terms, it is customary to allow cells to autolyze in heavy suspensions under a layer of toluene at room or incubator temperatures. To determine optimum conditions, a series of samples of cells may be suspended in buffers at a variety of pH's. The supernatants should be tested at intervals to determine the time of greatest activity. After such a survey, a standard practice for autolyzing particular cells may be adopted.

LYSIS BY ADDED AGENTS

Lysis can be induced readily by the addition of foreign agents to certain sensitive species of bacteria. A notable example is Micrococcus lysodeikticus which is rapidly lysed by the addition of raw egg white, saliva, or tears to a suspension of the organism. Fleming and Allison (1924) found that a heavy suspension could be completely cleared in 30 seconds at 50° C. by the addition of 1% egg white. On incubation for 24 hours at 37° lysis was observed in a one to 50 million dilution of egg white.

Penrose and Quastel (1930) compared the enzyme activity of intact and lysed cells of M. lysodeikticus, and reported that lysis increased the rate of p-phenylenediamine oxidation, increased the activity of catalase, fumarase, and urease, left peroxidase activity unchanged, and destroyed or reduced the activity of the dehydrogenases for glucose, fructose, lactic acid, succinic acid, glutamic acid, and glycerophosphoric acid. Krampitz and Werkman (1941) grew M. lysodeikticus on a glucose, peptone, yeast extract medium, and washed and recovered the cells by centrifugation. To a 10% suspension of wet cells they added 1/10 volume of saliva, and incubated the mixture for 1 hour at 36° C. The cell-free supernatant exhibited an active oxalacetic acid decarboxylase.

In his studies of formic acid decomposition by Escherichia coli, Stickland (1929) found that autolysis would not liberate the enzymes involved. He resorted to digestion of the cells at 37°, pH 7.6, with crude trypsin, using 5 ml. of Benger's "liquor pancreaticus" to 100 ml. of cell suspension. Periodic tests for dehydrogenase activity showed an initial rise followed by destruction of lactic and succinic dehydrogenases, whereas formic dehydrogenase activity continued to increase. The treatment yielded a cell-free formic dehydrogenase, whose activity appeared to be associated with cell debris, but completely destroyed formic hydrogenlyase and hydrogenase thus preventing the production of H_2 and CO_2 anaerobically or H_2O and CO_2 aerobically from formate.

Though we can cite no case of its having been used for bacterial enzyme preparations, Sylvester (personal communication) has suggested the possibility of inducing lysis by bacteriophage. The lytic action is rapid and the treatment mild, hence one might anticipate that enzymes would be liberated with little inactivation.

FREEZING AND THAWING OF CELLS

Bacterial enzymes often may be released into solution by rupturing the cell with alternate freezing and thawing. By such a method Avery and Neill (1924) prepared a cell-free extract of pneumococci that formed peroxide when exposed to oxygen. The cells from a broth culture were recovered by centrifugation and suspended in one volume of phosphate buffer or nutrient broth for each 35 volumes of the original culture. This suspension of unwashed cells was placed in long, narrow tubes, sealed with vaseline, and alternately frozen and thawed rapidly 6 to 9 times. The cell debris was sedimented by 3 or 4 high

speed centrifugations. The material did not lose its activity on passage through a Berkefeld filter under an atmosphere of nitrogen.

Koepsell and Johnson (1942), in their studies of the pyruvic acid metabolism of Clostridium butylicum, used a cell-free solution prepared by freezing the bacteria. Wet cell paste, as taken from the Sharples supercentrifuge, was packed in stoppered tubes and frozen immediately after harvest. While frozen, the cells slowly ruptured and released their contents. After 12 days 85 g. of cell paste was evenly suspended in boiled, cooled water, to give 250 ml. volume, and centrifuged. The supernatant liquid contained most of the original activity of the cells. This supernatant was dried under high vacuum and as a dry powder remained stable for some months.

COMMENTS

It has been our purpose to emphasize means of preparing cell-free juices which require relatively simple equipment, to stress the methods we have personally employed, and to describe briefly and cite references to other procedures. Unquestionably the Booth-Green mill and properly designed supersonic apparatus will yield excellent enzyme preparations from bacteria, but the equipment necessary is not widely available. The powdered glass grinding method of Wiggert, Silverman, Utter and Werkman (1940) and the modification described by Utter and Werkman (1942) has much to recommend it, because of its general application and because it involves only mild treatment. Dried cells are also widely applicable and are prepared readily by the methods described in the following section.

R. H. Burris

DRYING CELLS

This has proven to be one of the most efficient and convenient methods of preparing enzyme preparations from bacterial cells and a surprising number of enzymes have been found to be stable to the treatments described. Dried preparations are normally of two types: those obtained by acetone treatment and those obtained by lyophilization (drying from the frozen state in vacuo).

Acetone Preparations: These are similar to the preparations made from animal tissues. A bacterial suspension, usually a thick cream, is added dropwise to at least 10 volumes of ice cold, dry acetone which is vigorously stirred. When the entire suspension has been added it will usually flock and if stirring is discontinued the cells will settle rapidly. If flocking does not occur, more dry acetone should be added. After settling for 5-10 minutes, the supernatant is decanted off and the remainder filtered (usually with suction). The residue on the filter is treated with a small portion of dry, cold acetone which is sucked off as rapidly as possible. Some prefer to wash the preparation with alcohol or alcohol-ether mixtures but our own experience is that this is usually best omitted. The residue is spread on filter paper and dried either in air or under a slight vacuum.

Vacuum Dried Preparations: The bacteria are suspended in distilled water to form a heavy suspension. This should contain enough water so that it will flow freely and must be more dilute than a paste. Various methods for drying the cells are then used. In one, the suspension is placed in a flat dish in a desiccator over Drierite (or P_2O_5, $CaCl_2$, conc. H_2SO_4, etc.) and the desiccator evacuated with a rotary oil-sealed pump. The water is drawn off rapidly enough so that the cells freeze during the first five minutes of evacuation and subsequent drying is from the frozen state. Normally, one leaves the pump on for about 3-4 hours, and allows the preparation to remain under the vacuum in the desiccator for 8-10 hours more before opening. In another method, the cell suspension is first frozen (usually in dry ice) before the evacuation is begun. In a third, the suspension is frozen in a thin layer by rotating the container in a bath of dry ice ("shell dry") before the evacuation.

Use of Dried Preparations: Once obtained, the preparation is first tested for activity and if this is present it is likely to be stable for relatively long periods (especially if the preparations are kept cold and dry). However, the enzyme usually is not yet in

the cell-free state and permeability, while it may be altered, is sometimes not sufficiently different from the living cell to permit extraction of the enzyme. Autolysis may permit the extraction of the system one is interested in. However, when extraction procedures of this type do not work it is frequently possible to obtain the enzyme desired in a cell-free state by grinding the powder in vacuo. This is accomplished by a method developed by Gunsalus and Umbreit (1945) as follows:

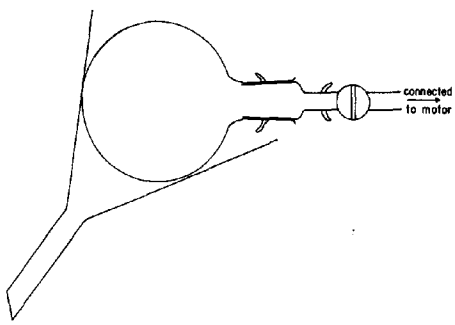

FIG. 49

Mill for grinding dry bacterial cells. The funnel may be plugged and filled with light oil to serve as a lubricant.

Into a round bottom flask with a ground glass neck are weighed 100-500 mg. of the dried cells. Glass beads of varying sizes are then added until the bottom surface of the flask is covered. The stopper containing a stopcock is inserted and the whole system evacuated with a rotary oil-sealed pump. The stopcock is closed, the flask removed from the vacuum pump and attached to a motor which rotates the flask slowly. A convenient support is a funnel containing a light oil as indicated in the figure. Rotation is at such a speed that the beads roll in the flask rather than tumble over each other as in the typical ball mill. The mill is allowed to run for about 8 hours (or overnight) by which time 90-95% of the cells will have been destroyed. The mill appears to break the cells largely by a shearing action and it apparently is effective because the beads become coated with cells and rotate rapidly. When contact with a similar rotating bead is achieved the bacterial cell wall apparently is ruptured by the forces involved. After grinding, the air is slowly let into the flask, the water, buffer, or extracting solution added and the suspension removed. It is clarified by centrifugation or settling. Numerous enzymes may be obtained from bacteria in this way.

PREPARATION OF MOLD AND ACTINOMYCES TISSUES

Both molds and actinomyces normally grow as a heavy mat over the surface of media. This mat may be handled in much the same manner as animal tissues. It may be removed, washed with water and cut into slices. Semeniuk (1944) has used a process roughly equivalent to the homogenate technique in which the mycelium was ground with sand. The use of the homogenizer itself (see Chapter 11) has not been, in our experience, very satisfactory since most of the activity of the tissue was lost and various supplements had little effect.

A second technique which is used with both molds and actinomyces (Woodruff and Foster, 1943) is to grow the mycelium in a submerged state with aeration. This is done either by forcing sterile air through the medium or by continual shaking of the culture flasks. In the latter the usual procedure is to use about 100 ml. of medium in a 250 ml. flask (Erlenmeyer) and to place the inoculated flask in a shaker which operates with a 2 to 4 cm. stroke at a speed of between 60 to 100 cycles a minute. Under these circumstances "pellets" of mycelium are formed which can be pipetted readily. One precaution should be noted; such pellets may not have the same metabolism as the mycelium grown on the surface. Knight (1948) has studied the l-amino acid oxidase of penicillin-producing molds in preparations of mycelium that were treated with acetone, dried and ground. Various preparations of mold and actinomyces tissue may also be employed in the study of phosphorylation. So far as present knowledge of phosphorylation extends it apparently differs from the known processes of animal tissues (Mann, 1943; Semeniuk, 1944), if, indeed, it exists in molds (Nord, Dammann and Hofstetter, 1936; Nord, 1939).

BUFFERS

It is assumed that the reader is familiar with the concept of pH. We wish here merely to develop the "pKa" concept and to show its usefulness.

Any acid, capable of ionization, when placed in an aqueous solution will liberate hydrogen ions according to the following equation:

$$HA \rightleftharpoons H^+ + A^-$$

or

$$(H^+)(A^-)/HA = K$$

If one takes the logarithms of both sides of the equation one has,

$$\log (H^+)(A^-)/HA = \log K$$

$$\log H^+ + \log A^- - \log HA = \log K$$

$$\log H^+ = \log K + \log HA - \log A^-$$

$$-\log H^+ = -\log K + \log A^-/HA$$

Under practical circumstances, any salt of the acid present will contribute A^- ions, hence

$$pH = -\log K + \left(\log \frac{\text{salt}}{\text{acid}}\right)$$

The term -log K may be expressed as pKa (Equation 50):

$$(50) \quad pH = pKa + \left(\log \frac{\text{salt}}{\text{acid}}\right)$$

It would thus appear from equation (50) that when the acid is half neutralized, the pH of the solution would be pKa. And when 10% of it were neutralized, roughly, (pH = pKa + log 1/10 = pKa -1) the pH would be 1 unit lower than the pKa; when 90% was neutralized, the pH is roughly 1 unit higher than the pKa. Over the range of physiological activity one can thus pick buffers which will maintain the pH relatively constant.

A base will dissociate as follows:

$$BOH \rightleftharpoons B^+ + OH^-$$

or

$$K_b = \frac{(B^+)\ (OH^-)}{BOH}$$

taking the logarithm of both sides:

$$\log K_b = \log B^+ + \log OH^- - \log BOH$$

$$-\log OH^- = -\log K_b + \log \frac{B^+}{BOH}$$

$$= -\log K_b + \log \frac{\text{salt}}{\text{base}}$$

-log OH may be expressed as pOH.

But since the dissociation constant of water is 1×10^{-14},

$$pH = 14 - pOH, \qquad \text{hence } pOH = 14 - pH$$

$$14 - pH = -\log K_b + \log \frac{\text{salt}}{\text{base}}$$

$$pH = 14 + \log K_b - \log \frac{\text{salt}}{\text{base}}$$

- log K_b can be designated as pK_b, hence this equation may be expressed as:

$$pH = 14 - pK_b - \log \frac{salt}{base}, \quad \text{or}$$

$$pOH = pK_b + \log \frac{salt}{base}$$

Some examples of the use of the pK_a and pK_b relationship are as follows:

1. Acetic acid: $K = 1.86 \times 10^{-5}$ and $pK_a = 4.73$; hence acetate-acetic acid mixtures would be suitable as buffers over the range 3.7 - 5.7 (representing 10 - 90% neutralization).

2. Similarly, for phosphoric acid:
   $H_3PO_4 \rightleftharpoons KH_2PO_4$; $K_1 = 1.1 \times 10^{-2}$; $pK_a = 1.959$; pH range, 1-3.
   $KH_2PO_4 \rightleftharpoons K_2HPO_4$; $K_2 = 2 \times 10^{-7}$; $pK_a = 6.7$; pH range, 5.7 - 7.7.
   $K_2HPO_4 \rightleftharpoons K_3PO_4$; $K_3 = 3.6 \times 10^{-13}$; $pK_a = 12.44$; pH range, 11.4 - 13.4.

3. For bases, ammonium hydroxide has a basic dissociation constant of 1.8×10^{-5} and a pK_b of 4.74. Ammonium hydroxide - ammonium salt mixtures would serve as buffers over the range 8.3 (14 - 5.74) to 10.3 (14 - 3.74). In the case of bases, the lower the numerical value of the dissociation constant, the lower the pH at which they serve as buffers.

In Table XXI are collected the dissociation constants, pK_a and pK_b values, and the pH at which they serve as buffers for a variety of acids and bases. This table should aid in the search for some material, other than the usual type of buffer, suitable for obtaining a particular pH.

The usual buffers are shown in Fig. 50; the data taken from Clark (1920) have been corrected to the currently accepted values for pH. The use of this chart is obvious but a few examples will serve to clarify the working details.

1. Desired: 0.05 M buffer of pH 3. Take 50 ml. of 0.2 M phthalate plus 21.5 ml. of 0.2 M HCl and dilute to 200 ml.

2. Desired: 0.05 M buffer of pH 5. Take 50 ml. of 0.2 M phthalate plus 22.5 ml. of 0.2 M NaOH and dilute to 200 ml.

3. Desired: 0.067 M phosphate buffer of pH 6.7. Take 6.0 ml. of 0.067 M KH_2PO_4 plus 4 ml. 0.067 M Na_2HPO_4.

4. Desired: 0.05 M buffer of pH 9. Take 50 ml. borate-KCl mixture plus 20 ml. 0.2 M NaOH and dilute to 200 ml.

One note of caution should be emphasized. The composition of the buffer is not to be disregarded in physiological experiments. One must take care that effects observed by altering the pH are due to change in hydrogen ion concentration and not to other alterations of the buffer.

W. W. Umbreit

TABLE XXI

Properties of Acids and Bases Suitable for Buffers

Acid or base (indented)	Dissociation Constant		pK_a	pK_b	Buffer at pH
Pyrophosphoric	K_1	1.4×10^{-1}	0.854		1
"	K_2	1.1×10^{-2}	1.959	-	2
Phosphoric	K_1	1.1×10^{-2}	1.959	-	2
o-Aminobenzoic		1.4×10^{-12}	-	11.854	2.1
Glycine		4.4×10^{-12}	-	11.647	2.3
α-Alanine		5.1×10^{-12}	-	11.293	2.7
Malonic	K_1	1.6×10^{-3}	2.88	-	3
Phthalic	K_1	1.26×10^{-3}	2.90	-	3
Tartaric	K_1	1.1×10^{-3}	2.96	-	3
Salicylic	K_1	1.06×10^{-3}	2.98	-	3
Fumaric	K_1	1×10^{-3}	3.0	-	3
Citric	K_1	8.0×10^{-4}	3.1	-	3
Sulfanilic		6.2×10^{-4}	3.22	-	3
Brucine	K_2	2.5×10^{-11}	-	10.602	3.5
Mandelic		4.29×10^{-4}	3.36	-	3.5
Malic		4.0×10^{-4}	3.39	-	3.5
Hippuric		2.3×10^{-4}	3.64	-	3.5
Formic		1.76×10^{-4}	3.76	-	4
Lactic		1.38×10^{-4}	3.86	-	4
Barbituric		1.05×10^{-4}	3.98	-	4
Tartaric	K_2	6.9×10^{-5}	4.16	-	4.2
Succinic	K_1	6.6×10^{-5}	4.18	-	4.2
Oxalic	K_2	6.1×10^{-5}	4.21	-	4.2
Quinine	K_2	3.3×10^{-10}	-	9.481	4.5
Fumaric	K_2	3×10^{-5}	4.52	-	4.5
Acetic		1.86×10^{-5}	4.73	-	4.7
Citric	K_2	1.8×10^{-5}	4.75	-	4.7
Aniline		4.6×10^{-10}	-	9.337	4.8
Valeric		1.6×10^{-5}	4.80	-	4.8
Butyric (n-, iso-)		1.48×10^{-5}	4.83	-	4.8
Propionic		1.4×10^{-5}	4.86	-	4.9
Methylamine		1×10^{-5}	-	9.0	5.0
Quinoline		1×10^{-5}	-	9.0	5
Malic	K_2	9×10^{-6}	5.05	-	5.1
Benzoic		6.3×10^{-6}	5.21	-	5.2
p-Toluidine		2×10^{-9}	-	8.70	5.3
Pyridine		2.19×10^{-9}	-	8.66	5.3
Citric	K_3	4×10^{-6}	5.40	-	5.4
Phthalic	K_2	3.1×10^{-6}	5.51	-	5.5
Succinic	K_2	2.8×10^{-6}	5.56	-	5.6
Malonic	K_2	2.1×10^{-6}	5.68	-	5.7
Uric		1.5×10^{-6}	5.83	-	5.8
Carbonic	K_1	3×10^{-7}	6.53	-	6.5
Pyrophosphoric	K_3	2.9×10^{-7}	6.54	-	6.5
Maleic	K_2	2.6×10^{-7}	6.58	-	6.6
Phosphoric	K_2	2×10^{-7}	6.7	-	6.7
Strychnine		1×10^{-7}	-	7.0	7.0
Quinine	K_1	2.2×10^{-7}	-	6.6	7.3
Pyrophosphoric	K_4	3.6×10^{-9}	8.44	-	8.4
α-Alanine		9×10^{-10}	9.10	-	9.1
Boric	K_3	6.4×10^{-10}	9.20	-	9.2
Glycine		1.66×10^{-10}	9.78	-	9.8
Phenol		1.3×10^{-10}	9.89	-	9.9
Ammonium hydroxide		1.8×10^{-5}	-	4.74	9.3
Dimethylamine		3.6×10^{-5}	-	4.44	9.6
Butylamine (sec.)		4.4×10^{-4}	-	3.36	10.6
Methylamine		5×10^{-4}	-	3.30	10.7
Dimethylamine		5.2×10^{-4}	-	3.29	10.7
Ethylamine		5.6×10^{-4}	-	3.25	10.7
Brucine	K_1	7.2×10^{-4}	-	3.14	10.8
Phosphoric	K_3	3.6×10^{-13}	12.44	-	12.4
Salicylic	K_2	1×10^{-13}	13.0	-	13.0

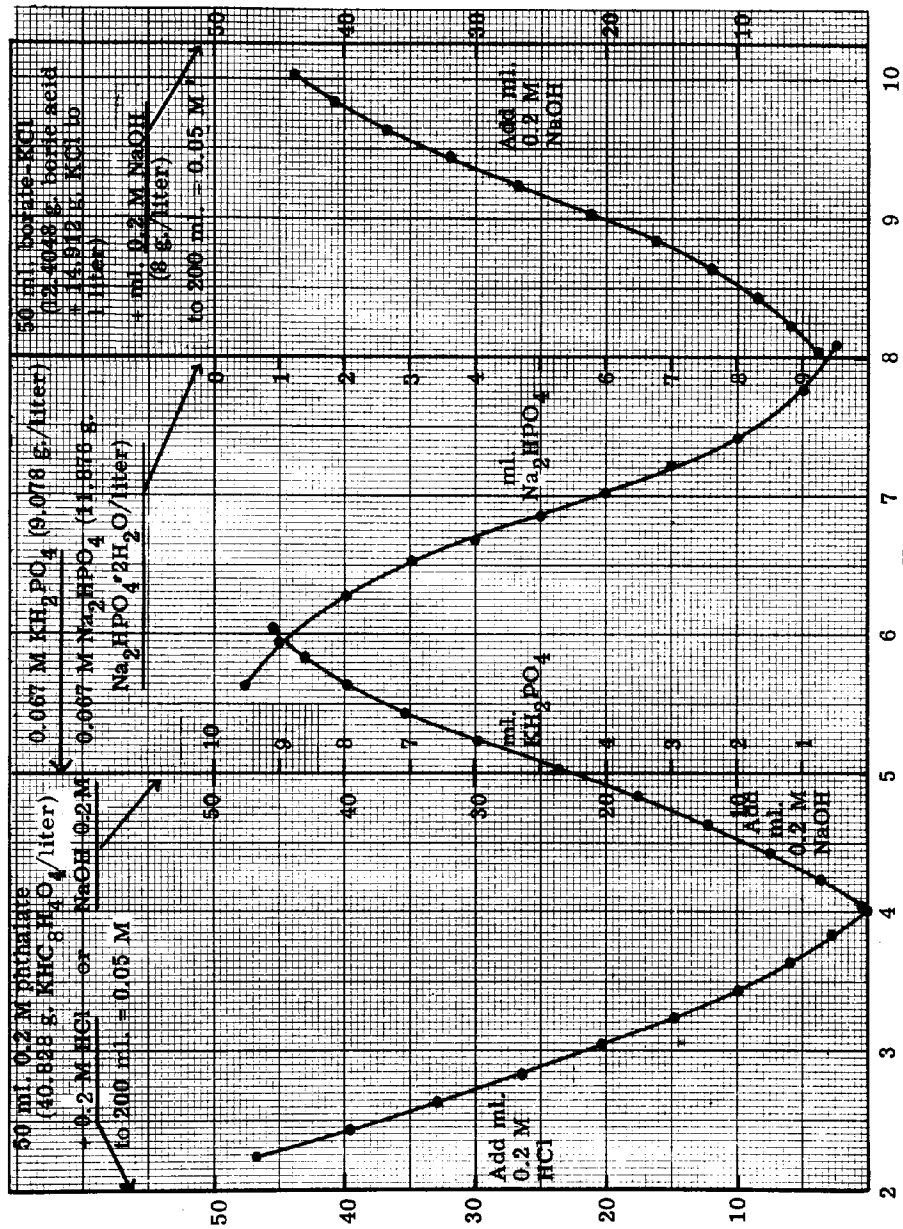

FIG. 50

Graph indicating composition of buffers.

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Chapter XI

THE HOMOGENATE TECHNIQUE

PRINCIPLES

The use of homogenates assumes for purposes of methodology that any chemical reaction which occurs in living cells will also occur in cell-free preparations of protoplasm; that this assumption is not unjustified has become evident during the years since publication of the original paper (Potter & Elvehjem, 1936). Homogenates consist of suspensions of the particulate components of protoplasm including mitochondria, microsomes (Claude, 1943), secretory granules, etc. plus a solution of the freely soluble diffusible components of protoplasm such as inorganic ions, coenzymes and carriers. If such preparations are properly made it is possible to dilute them until the endogenous oxygen uptake is virtually abolished, and then by the restoration of appropriate soluble factors at the proper concentration it is possible to study the reactions of any enzyme desired, to the exclusion of other enzymes whose cofactors or substrates have been eliminated by dilution. The method was devised in order to be able to assay quantitatively specific enzymes in the tissues of experimental animals. It was necessary to develop methods which would be applicable to whole tissue homogenates rather than to any extracts thereof, since any extraction method would be inexact because the completeness of extraction would be unknown. The homogenate methods are therefore designed to isolate a particular reaction, and, by so doing, to determine quantitatively the specific enzyme which catalyses the reaction. The methods have been applied to tissue samples as small as 13 mg. (immature rat ovaries) and are adapted to rapid manipulation. It remains to be seen how many enzymes can be studied by this technique, but several applications will be described.

In addition to the assay methods, the homogenate technique lends itself to the study of reaction mechanisms which involve a number of coordinated enzyme systems, and to the development of procedures for the testing and fractionation of new enzymes. By adding certain cofactors and substrates and omitting others it is possible to effect a reconstruction and integration of particular enzyme organizations which represent segments of cell function.

CONSTRUCTION OF THE HOMOGENIZER

The apparatus consists of a test-tube and a close-fitting power-driven pestle (see Fig. 51). The original homogenizer consisted of a 16 x 150 mm. pyrex test tube and a pestle which was made by sealing off one end of a 220 mm. length of 6 mm. capillary tubing and blowing a thick-walled cylindrical bulb about 20 mm. long, using a slightly larger test-tube for mold. Later it was found advantageous to form the bulb from a piece of thick-walled glass tubing whose outside diameter is that of the inside of the test-tubes. The large tubing is sealed to the capillary tubing, then constricted and sealed off at a length of about 20 mm., and molded in the test tube as before. For working with extremely small tissue samples, it is convenient to use small homogenizers, in which case 13 x 100 mm. pyrex tubes can be used, and the pestles can be blown from 6 mm. capillary tubing as in the original method. The final operation is the sealing of 6 or 7 small beads of about 2 mm. diameter to the bottom of the pestle to form cutting teeth. The device is then ready to grind. The beads are ground down on emery cloth so that each one has a flat surface which will approximate the inside of the bottom of the test tubes. This gives them a right-angled cutting edge which remains until the teeth disappear with continued use. Pestles with teeth less than 1 mm. high are best. It is desirable to have five or ten outfits on hand and to have a number of test tubes for each pestle. The sides of the pestles are ground with a few moments operation in an over-size test-tube containing a light suspension of fine carborundum powder in water. The tubes are ground similarly, using

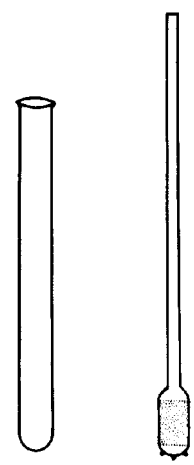

FIG. 51

Tissue Homogenizer.

an undersize pestle. The object is not to produce a ground glass surface over the entire area of the grinding surfaces, but rather to eliminate all of the irregularities from both the tubes and the pestles. When the grinding is completed, pestles and tubes are matched by testing them with water in the tubes: the tubes should fall off the pestles very slowly when not supported. The clearance is then 0.10 - 0.12 mm. For any given pestle, it is well to have both tight-fitting and loose tubes, and as wear takes place, new tubes can be fitted to the pestle. (The presence of powdered glass in the homogenates can introduce serious errors into dry weight determinations where these are made by evaporating aliquots of dilute homogenates. M. O. Schultze has found that Lucite can be used to construct pestles, as it can be machined to any desired dimension. It was said that Lucite pestles do not erode glass from the test-tubes and that the resulting homogenates yield valid dry weight data.)

In Cori's laboratory, the pestles have been made from stainless steel. We also use stainless steel pestles for certain operations such as grinding tissues in trichloracetic acid for analytical purposes, in which case loose fitting tubes are used. The bulb of the pestle was machined to size and threaded so that a 6 mm. threaded rod could be screwed into it. The bottom of the pestle was scored to form cutting teeth.

The homogenizer is powered by a cone-driven stirring motor of the Cenco or Sargent type, operated at a speed of about 1000 r.p.m. The use of a cone-drive motor prevents breakage because when a piece of connective tissue or muscle jams between the pestle and the tube, the friction drive slips instead of breaking the pestle.

Unground glass pestles may be obtained from Erway Glassblowing Company, Oregon, Wisconsin.

THE USE OF THE HOMOGENIZER

Homogenates are usually not prepared until the reaction mixtures have been added to all of the vessels. The animal is usually killed by decapitation and the desired organ is quickly excised and placed in cold isotonic medium in a small beaker surrounded by cracked ice, as a general procedure. For assay purposes, a representative tissue sample should be taken. This is no problem in the case of most organs such as liver or muscle, but in the case of kidney and brain it is best solved by using one whole kidney or a specified lobe of the brain. Unless the amount of tissue is limited, it is well to take a sample weighing between 500 and 800 milligrams wet weight. The tissue can be accurately and speedily weighed on a torsion balance of one gram capacity graduated in fifths of a milligram, such as is made by the Roller-Smith Company.

The weighed tissue is dropped into a homogenizer tube which contains 1 ml. of medium, which may be distilled water, isotonic KCl, isotonic sucrose or some other solution that will be determined by the needs of the assay. In recent work an alkaline isotonic KCl has been used. This consists of 0.154 M KCl plus 8 ml. of 0.04 M $KHCO_3$ per liter. The final homogenate should have a pH of about 7.0. To make a ten per cent homogenate it is simplest merely to add 9 times the weight of the tissue (minus the one ml. which was originally in the tube). Very often more dilute homogenates are used. These are conveniently made by diluting an appropriate amount of 10 per cent homogenate, without further grinding. For some work it is unnecessary to chill the tissue, the homogenizer and the medium. However, it is desirable in many cases and we find it convenient to keep the motor in the cold room, while the homogenizer tube is kept in cracked ice when the tissue is being weighed and the calculations are being made.

The homogenizer functions by tearing up the tissue with the cutting teeth and then grinding it between the walls of the tube and the walls of the pestle. Accordingly, the tube is moved up and down rapidly in order to force the tissue back and forth past the grinding walls. The pestle is supported rigidly in a chuck on the drive shaft of the stirrer. Various investigators have used a short length of pressure tubing to effect a universal joint between the drive shaft and the pestle. This is not satisfactory for the production of cell-free homogenates unless the homogenizer tubes and pestles are almost perfectly matched. On the other hand, imperfectly fitting tubes and pestles can be used quite successfully when the pestle is supported rigidly, if the operator pushes the tube

laterally while working it up and down so that the clearance between pestle and tube is zero on one side. One has to avoid breaking the pestle, of course.

The completed homogenate will be relatively cell-free but will contain shreds of connective tissue which tend to clog ordinary pipettes. It is advisable to use pipettes that have slightly enlarged openings at the end for pipetting homogenates. These are easily prepared when the tips are drawn out, an operation which should be done with all pipettes used for this type of work anyway. Mohr pipettes are used.

Homogenates should not be stored and used later on in the same day unless this fact is recorded with the data. Many enzymes which have been too labile to study thus far by other techniques can be studied by means of fresh homogenates.

PREPARATIONS

Thus far all the respiratory enzyme systems which have been studied by the homogenate technique have been found to require the addition of cytochrome c and it should be added whenever these systems are being investigated. Indeed, one of the main experimental developments which made it possible to develop the use of homogenates was the preparation of cytochrome c by Keilin and Hartree (1937) which is described in Chapter 16, page 211.

Cytochrome c can become limiting in a homogenate, and can be added back to the reaction mixture to make other biocatalysts the rate-determining factors. If they can be obtained free from the enzymes under assay, other enzymes that are soluble can be similarly used. They also must be free from enzymes that are damaging to the enzyme systems under assay. It is possible to prepare solutions of enzymes free from the particulate components which function in the electron transmitting systems. These solutions contain the enzymes of glycolysis and are sometimes called Meyerhof extracts. They are also rich in the various coenzyme I dehydrogenases (Green, Needham and Dewan, 1937; Dewan and Green, 1938). Such preparations have been used to fortify homogenates in the study of triosephosphate dehydrogenase (Potter, 1940) and for the study of malic dehydrogenase and the coenzyme I-cytochrome c reductase (Potter, 1944). We have followed the method of Green, Needham and Dewan and found it satisfactory. Description of the preparation of these materials will be found in Chapter 16, page 218.

The study of interrelations between the respiratory enzymes and the phosphorylation systems can be studied conveniently by means of homogenates, provided the necessary components are present. One of the compounds that is most frequently needed is adenosinetriphosphate (ATP), because it is the common denominator for so many of the interlocking reactions of phosphorylation respiration. Furthermore, the compound forms the starting material for the preparation of its derivatives, which include adenosinediphosphate (ADP) and adenosine-5-monophosphate (adenylic acid), and the inosine series of compounds. Until recently these compounds could not be purchased but several have now been placed on the market by several commercial organizations, including Schwartz Laboratories, Rohm and Haas, Ernst Bischoff, and Armour and Co. These preparations vary somewhat in purity and should be tested at various levels in biological systems. Slight metallic impurities have the effect of decreasing the activity of an enzyme system when increasing amounts of nucleotide are added, in contrast to results obtained with preparations of high purity in which a broad plateau of maximum activity is observed. If the amount of homogenate added is varied, a plot of rate *vs.* homogenate yields a straight line passing through the origin with pure preparations. With impure preparations the line intersects the homogenate axis. The methods for preparing ATP have been given by Lohmann and Schuster (1935) and by Needham (1942). Its preparation is described in Chapter 16, page 204.

Of the remaining substances that are needed for investigations involving homogenates, little can be said except to emphasize purity. We have obtained Merck products such as ascorbic acid, cysteine and other amino-acids whenever this has been possible. Merck also has been able to provide cocarboxylase. Pyruvic acid obtained from any source must be redistilled at 3-5 mm. Hg, preferably twice (Lipschitz, Potter and Elvehjem, 1938). The product should be diluted to approximately 1 Normal (addition of about 17 volumes of water; Sp. Gr. of pyruvic acid is 1.288). The diluted acid should be standardized, and adjusted immediately before using, by the addition of the theoretical amount of bicarbonate solution to a diluted pyruvic acid to give a final concentration of 0.1 N, and the pH should

never rise above 6.8 during the neutralization. Robertson (1942) has purified pyruvic acid by precipitating the sodium salt from alcohol, see page 208. Potter and Schneider (1942) purified sodium succinate ($\cdot 6H_2O$) by alkaline precipitation of metals and recrystallization. Malic acid can be obtained from Pfanstiehl or Eastman. Citric acid is readily available but iso-citric acid is not. However, Pucher, Abrahams and Vickery (1948) have obtained the latter from Bryophyllum leaf tissue. Hexosediphosphate and DPN (diphosphopyridine nucleotide, cozymase, coenzyme I) can be obtained from Schwartz Laboratories, Inc., and glutathione from the B. L. Lemke Company.

RESPIRATORY ENZYMES

Succinic dehydrogenase and cytochrome oxidase: The technique for assaying animal tissues for these two enzymes was described by Schneider and Potter (1943). The assay for cytochrome oxidase was carried out on the same tissue samples as the assay for succinic dehydrogenase, and it was found that in all cases the oxidase was present in wide excess over the dehydrogenase. This means that when succinate is the substrate the limiting factor is the enzyme (or enzymes) which oxidize succinate and reduce cytochrome c, that is, succinic dehydrogenase. On the other hand, when cytochrome c is reduced chemically, the limiting factor is the enzyme (or enzymes) which brings about the reaction between cytochrome c and oxygen, that is, cytochrome oxidase. Although both enzymes may consist of more than one component they may be treated as single entities for assay purposes. The sequence of reactants in the two cases shows the relation of the two assays.

Succinic Dehydrogenase assay: (Rat Liver $Q_{O_2} = 88$)

Succinate ⟶ Succinic Dehydrogenase ⟶ Cytochrome c ⟶ Cytochrome Oxidase ⟶ O_2

Cytochrome Oxidase assay: (Rat Liver $Q_{O_2} = 392$)

Ascorbate ⟶ Cytochrome c ⟶ Cytochrome Oxidase ⟶ O_2

In every tissue thus far examined the preponderance of cytochrome oxidase over succinic dehydrogenase has been great enough to make the succinic dehydrogenase assay a valid one. One would expect the cytochrome oxidase system to have a greater capacity for electron transfer than any one of the systems which funnel into it, and the fact that such is the case makes it technically possible to measure the capacity of the component systems. The assay has been arranged so that one sample of tissue can be assayed for both enzymes using six flasks and manometers set up as follows, using rat liver as an example:

TABLE XXII

Reaction Mixtures and Results in the Succinic Dehydrogenase-Cytochrome Oxidase Assay (37°C.)

The center cups contained 0.2 ml. 2N NaOH + 3 Sq. cm. folded filter paper; Warburg flasks without sidearms; 10 minutes equilibration. Reactants and homogenates at room temperature prior to assay.

Flask No.	1	2	3	4	5	6
	ml.	ml.	ml.	ml.	ml.	ml.
H_2O (to make 3.0 ml.)	0.6	0.5	0.9	0.3	0.25	0.20
0.1 M PO_4 pH 7.4 with NaOH	1.0	1.0	1.0	1.0	1.0	1.0
0.5 M Na-Succinate pH 7.4	0.3	0.3	0.3	--	--	--
1 x 10^{-4} M. Cytochrome c	0.4	0.4	--	--	--	--
2.4 x 10^{-4} Cytochrome c	--	--	--	1.0	1.0	1.0
4 x 10^{-3} M. $CaCl_2$	0.3	0.3	0.3	--	--	--
4 x 10^{-3} M. $AlCl_3$	0.3	0.3	0.3	0.3	0.3	0.3
0.114 M. Na-ascorbate pH 7.0.*	--	--	--	0.3	0.3	0.3
1% rat liver homogenate in water **	--	--	--	0.10	0.15	0.20
5% rat liver homogenate in water **	0.1	0.2	0.2	--	--	--
µl. O_2 uptake per 10 min. (average of four 10 minute periods)	20.0	42.0	8.0	30.4	43.2	56.0

*Prepared by adding 1 ml. of 0.1 N NaOH to 20 mg. of ascorbic acid just before use.
**Diluted from 10 per cent homogenate.

The plan does not include a measurement of the endogenous oxygen uptake of the liver because experience has shown that this is insignificant. However, it is well to establish this point in any new work, especially if larger amounts of tissue are used. The amount of tissue is usually chosen so as to give oxygen uptake values of approximately the range indicated. The treatment of the data will now be described:

Succinic dehydrogenase: The necessary data are given by flasks 1 and 2. The assay is always run at two levels of tissue rather than with duplicates at the same level, because this technique has the advantage of providing continual proof that the uptake is proportional to the tissue concentration. The data are reported finally as Q_{O_2}, that is, the microliters of oxygen taken up per hour per milligram of dry weight. However, the data are first converted to the 10/20 ratio, which is the average oxygen uptake per 20 mg. of fresh tissue per 10 minutes (or 10 mg. fresh tissue per 20 minutes). Since the readings are taken at 10-minute intervals and the homogenates are prepared in strengths of 1, 5, or 10% the 10/20 ratio can usually be calculated mentally, and its usefulness lies in the fact that it is identical with the Q_{O_2} when the per cent dry weight of the tissue is 30%. This is the value most frequently found for rat liver. To illustrate, the data from flasks 1 and 2 are used to obtain the 10/20 ratio and to calculate the Q_{O_2}:

$$\text{The 10/20 ratio} = \left[(4 \times 20) + (2 \times 42)\right] / 2 = 82$$

$$\text{The } Q_{O_2} = 82 \times 6 \times 1/20 \times 100/30 = 82$$

When the per cent dry weight is not 30, the 10/20 ratio is multiplied by $\frac{30}{\text{per cent dry wt.}}$ to give Q_{O_2}. The Q_{O_2} should be defined according to the substrate which is used. The data from flasks 1 and 2 therefore give the succinate Q_{O_2}.

Cytochrome oxidase: The cytochrome oxidase assay is complicated by the fact that the substrates are all more or less autoxidizable and are generally affected by traces of copper or catalysts other than the cytochrome system. We selected ascorbic acid as the best reductant for the system, but it also has some autoxidation, the measurement of which cannot be made simply by putting the substrate in a flask with the buffer and measuring the rate of oxidation. We have found that the best way to measure the autoxidation rate is to extrapolate to zero tissue concentration from a series of three different tissue concentrations as in the table, in which flasks 4, 5 and 6 contain 1.0, 1.5 and 2.0 mg. of fresh liver, respectively. The autoxidation rate is obtained in this case by substrating the increments in the last two flasks from the value of the first flask:

$$30.4 - (12.8 + 12.8) = 4.8$$

This value is then subtracted from all the values, and the 10/20 ratios are calculated. The oxygen uptakes corrected for autoxidation are then, respectively, 25.6, 38.4 and 51.2 and the 10/20 ratios are 256, 256 and 256 and the ascorbate Q_{O_2} is therefore 512.

In the paper by Schneider and Potter (1943) this figure was accompanied by a correction factor that was based on what is now referred to as a "cytochrome quotient" (see Schneider, Claude and Hogeboom, 1948). The correction was based upon the observation that the oxidation of ascorbate was more rapid in the conditions which decreased the oxidation rate on succinate in the absence of cytochrome c. Such conditions appear to cause the disruption of the mitochondria and the phenomenon may mean that the cytochrome oxidase in intact mitochondria is less available to ascorbate and cytochrome c than is the oxidase in disrupted mitochondria. It is probably appropriate to use the technique that gives the maximum rate and to apply no correction, although further studies need to be made.

The conditions which have been described as optimum for this enzyme system probably apply to other tissues as well, but should not be assumed to do so.

Malic dehydrogenase and DPN-Cytochrome C-reductase: It appears likely that these two enzyme systems can be assayed by utilizing the cytochrome system as the terminal connection with oxygen, since the cytochrome oxidase seems to be present in considerable excess (see preceding section). Handler and Klein (1942) showed that DPN is rapidly broken down

in homogenates and one might expect that the study of coenzyme systems might be impossible in homogenates. However they also showed that the breakdown is inhibited by nicotinamide, as had been reported by Mann and Quastel (1941). Oxalacetate, the product of malate oxidation, powerfully inhibits the reaction and unless it is removed the system cannot be studied. Its oxidative removal, besides being difficult to accomplish, would complicate the results. The oxalacetic acid is therefore removed by means of the transaminase reaction, which Straub (1941) utilized for this purpose. Transaminase is present in the homogenate. The reaction sequence is:

Malate-malic dehydrogenase-DPN---DPN-cytochrome c-reductase--cytochrome c--cytochrome oxidase--oxygen.

The underlined reagents are added in excess; the side reactions are controlled by adding nicotinamide and glutamate; the limiting factor is malic dehydrogenase when the cytochrome reductase is present in excess. In order to assay for the reductase, one must add malic dehydrogenase in excess; this can be done by adding a Meyerhof extract, the preparation of which has been described (page 138). The system appears to require a higher concentration of cytochrome c than does the succinate system. An assay can be carried out for both enzymes in duplicate using 4 flasks and manometers as follows, with no controls described. The removal of oxalacetate is still not 100% efficient and this probably accounts for the rapid slowing of the reaction. The data are therefore based on the first two five-minute readings, while a third reading is taken to show that the reaction has not slowed appreciably in the first fifteen minutes. The system has been studied in rat liver, which is the only tissue for which an attempt has been made to describe optimum conditions (Potter, 1946). The system probably could be improved further by adding purified transaminase (Schlenk and Fisher, 1947) and malic dehydrogenase (Straub, 1942). With the development of the glycolytic reaction system (see below), a better cytochrome reductase system might be developed using this system to reduce DPN instead of using the malic system. A spectrophotometric method for DPN-cytochrome c reductase has been used by Potter and Albaum (1943) but has not been developed as an assay.

TABLE XXIII

Reaction Mixtures in the Malic Dehydrogenase and DPN-Cytochrome c Reductase Assay (37°C.)

(0.2 ml. 2N NaOH + 3 sq. cm. filter paper in center cup)				
Flask No.	1	2	3	4
	ml.	ml.	ml.	ml.
H_2O	0.7	0.6	0.1	--
0.1 M. PO_4 pH 7.4 with NaOH	0.8	0.8	0.8	0.8
0.1 M. Nicotinamide	0.3	0.3	0.3	0.3
0.5 M. Na-malate	0.3	0.3	0.3	0.3
0.5 M. Na-glutamate	0.3	0.3	0.3	0.3
0.5% DPN (sidearm; added after equilibration)	0.2	0.2	0.2	0.2
4×10^{-4} M. cytochrome c	0.3	0.3	0.3	0.3
Meyerhof extract, excess	--	--	0.6	0.6
5% rat liver homogenate	0.1	0.2	0.1	0.2

The malate Q_{O_2} is calculated from flasks 1 and 2 and the DPN Q_{O_2} is calculated from flasks 3 and 4; the 10/20 ratio is based on the first ten minutes of oxygen uptake.

Keto-acid oxidases: The oxidation of pyruvic acid, oxalacetic acid, and α-ketoglutaric acid may be accomplished in homogenates that are fortified with magnesium ions and ATP. It is possible to use AMP also, since it is converted to ATP in such systems. It

is not a simple matter, however, to carry out one-step oxidations or to name the rate-limiting enzyme at this time, since these oxidations involve other intermediates in the Krebs tricarboxylic acid cycle, and depend upon a maintained level of ATP. Unlike the succinoxidase cytochrome oxidase and malic dehydrogenase systems, which can be studied in water homogenates, the keto-acid oxidations thus far appear to require isotonic homogenates for best results. The system has been studied in homogenates by Potter, LePage and Klug (1948) and by Potter, Pardee and Lyle (1948). The components of the system as employed by the latter investigators is given in Table XXIV.

TABLE XXIV

Standard Reaction Mixtures for the Oxidation of Keto-acids* (37°C.)

(0.2 ml. 2 N NaOH + 3 sq. cm. filter paper in center cup; Warburg flasks without sidearms; all reactants and flasks kept in ice bath prior to addition of homogenates.)

Addition	Volume
H_2O	0.7 ml.
0.5 M KCl	0.4
0.1 M $MgCl_2$	0.1
4×10^{-4} M cytochrome c	0.1
0.1 M K phosphate**	0.2
0.01 M ATP, K salt	0.3
0.0267 M K oxalacetate***	0.3
0.154 M KCl	0.2 to 0.7
10 per cent isotonic KCl homogenate	0.7 to 0.2

*This reaction mixture gives optimum rates of oxygen uptake for short periods of time with rat kidney homogenates. It should be modified depending on the objective, see text.

**May be combined with the preceding 4 components into a stock solution that is adjusted to pH 7.0 - 7.2.

*** Three-fourths neutralized with K_2CO_3 and aerated; 0.02 M K_2CO_3 is added to solid oxalacetic acid.

The conditions given in the table gave maximum rates of oxygen uptake with homogenates of liver as well as kidney. However in a study by Pardee and Potter (1949) it was found that brain and heart homogenates gave increased rates of oxygen uptake when a mixture of oxalacetate and pyruvate were used, suggesting that the conversion of oxalacetate to pyruvate may be limiting in these tissues. Experiments with malonate showed that in liver homogenates, pyruvate has two alternative oxidative pathways. In the presence of malonate, pyruvate can be oxidized to acetoacetate, while in the absence of malonate, it can evidently be oxidized through the Krebs cycle if a continuing supply of oxalacetate is present. The other tissues studied do not appear to possess the alternative pathway that leads to acetoacetate formation from pyruvate.

Thus the conditions described in Table XXIV may be varied considerably as to substrate. If pyruvate is substituted for oxalacetate, the amount can be increased to give a final concentration of about 0.005 M. However, the results with pyruvate alone have very little significance in tissues other than liver, since the rate will be determined by the uncertain amounts of C_4 acids that may be available. If this amount of pyruvate is used, maximum rates of oxidation can be obtained by adding oxalacetate, fumarate and probably malate. If succinate is added, the results are confused by the fact that this compound can be oxidized at a rate that is independent of the other reactions in the cycle. Although we have done comparatively few experiments with α-ketoglutarate, it appears that this compound can be used as a substrate with no other substrates present, and

from the work of Ochoa (1944) it should be possible to isolate the reaction by adding malonate if the findings of Pardee and Potter (1949) are kept in mind. The over-all reaction system is similar to the "cyclophorase" system of Green, Loomis, and Auerbach (1948) but their results were not expressed in terms of rates and were not designed to assay tissues for enzyme content. With the use of whole homogenates it should be possible to determine the amount of an individual enzyme in the system if it can be established as the rate-limiting component. It appears that various keto-acid oxidations can be made the rate-limiting steps by using the various combinations: (1) pyruvate + malonate (2) pyruvate oxalacetate or fumarate, and (3) α-ketoglutarate + malonate. However, in each case the rate-limiting reaction might be catalysed by any one of several enzymes. The following interpretation forms a basis for further work. In case (1) the reaction probably involves a pyruvic dehydrogenase and its electron transferring enzymes plus an enzyme for condensing C_2 groups to yield acetoacetate. The latter enzyme appears to occur in liver and to a very limited extent if at all in other tissues.

In case (2) the same pyruvic dehydrogenase and electron transport system are probably involved, but the C_2 fragment is perhaps utilized by an enzyme that condenses it with oxalacetate to yield a precursor of isocitrate. The measurements are complicated by the fact that the formation of isocitrate sets off the whole sequence of Krebs cycle oxidations.

In case (3) the condensation reactions do not appear to be involved, and in the presence of malonate the reaction probably involves only the α-ketoglutaric dehydrogenase and the electron transport system.

In all three cases one might expect that in normal tissues, the electron transport system would not be the limiting component, and the rate-limiting enzyme seems likely to be either the dehydrogenase or the enzyme that catalyzes the condensation. However, all of these reactions involve phosphorylation and if the dehydrogenation, phosphorylation and condensation reactions are inseparable, the assay becomes technically simplified and the rate of oxygen uptake might be an adequate measure of the enzyme complex. This outcome appears unlikely however, in view of the fact that Schneider and Potter (1949) have been able to separate homogenates by centrifugal means into fractions that gave lower rates of oxygen uptake when taken separately than when they were combined. These observations indicate that the enzyme components are not all in one particle. From the fact that the mitochondrial particles could be supplemented by supernatant fractions that were inactive alone it should be possible to fractionate the latter and prepare concentrates that could be used to elicit the maximum activity of the particles.

The final point to be emphasized is that the oxygen uptake data can be supplemented with analytical determinations of keto-acid disappearance and product formation to considerable advantage.

Glycolytic Enzymes: Glycolysis has been studied extensively in tissue slices and in tissue extracts, but in neither instance does one obtain a measure of the glycolytic enzyme content of the tissue sample. In slices, the aerobic enzymes mask the results of glycolysis when oxygen is present, and the glycolytic systems in many normal tissues appear to be unable to keep pace with ATP breakdown when oxygen is absent. Whatever the explanation, anaerobic glycolysis is very low in slices of such tissues (cf. Burk, 1939). On the other hand, in extracts one cannot be assured that the enzyme content of the extract is an adequate measure of the enzymes in the original tissue. Extracts played a decisive role in the elucidation of the nature of glycolysis, but they have never been proposed as the basis for tissue assays.

Homogenates were used originally for the study of glycolysis in brain. Further studies on the development of assay techniques have been made by LePage (1948) who reported on studies with cancer tissue, and by LePage and Schneider (1948) who measured glycolysis in fractions obtained by the centrifugation of homogenates. References to the earlier papers are given in these papers. The latter studies revealed that no fraction, including the final supernatant extract, was as active as the whole homogenate.

The optimum conditions reported by LePage (1948) are given in Table XXV.

TABLE XXV

Fluoride-Containing Reaction Mixture for Glycolysis in Homogenates (37°C.)

Addition	ml. per Flask
H_2O	0.75
0.024 M K-PO_4 pH 7.6	0.3
0.5 M $KHCO_3$	0.15
0.4 M Nicotinamide	0.3
0.01 M K-ATP	0.1
0.2 per cent K-DPN (calc. from assay value)	0.2
0.04 K-HDP	0.15
0.10 M Glucose	0.3
0.10 M $MgCl_2$	0.2
0.15 M K-pyruvate	0.10
0.20 M KF	0.15
10 per cent KCl homogenate	0.30

These conditions differ somewhat from the conditions given in an earlier review (Potter, 1948) but each of the essential additions is added at a level such that small variations in amount do not affect the glycolytic rate. The conditions given were worked out using Flexner-Jobling tumors as the source of enzyme. Further studies using various normal tissues are in progress and support the general conclusion of other studies relative to methodology; the optimum conditions for one type of tissue are likely to reveal activity if it is present in other tissues but are not necessarily the optimum for all tissues.

Thus far the studies on the glycolytic enzyme system have not established one enzyme as the rate-limiting factor. Under the conditions employed, there may be an initial rate-limiting step below hexose diphosphate which may later shift to the reactions involved in the conversion of glucose to hexose diphosphate. By carrying out analyses at various time intervals considerable information can be obtained regarding the relative amounts of the enzymes that are involved in glycolysis. Future studies on homogenates supplemented with individual enzymes may make it possible to establish the identity of rate-limiting steps, while alterations in the reaction mixture, such as increasing the HDP, may serve to eliminate certain steps. In all such studies the determination of inorganic phosphate and lactic acid as carried out by LePage is preferable to the measurement of CO_2 output alone.

THE COUPLING OF OXIDATION AND PHOSPHORYLATION

Adenosinetriphosphatase: The logical preliminary to the study of the coordinated phosphorylative and oxidative systems was a knowledge of the rate of dephosphorylation of the phosphorylated compound which has thus far been the immediate product of the phosphate donating reactions, that is, adenosinetriphosphate. DuBois and Potter (1943) devised an assay and applied it to normal rat tissues. It was found that the calcium ion was a necessary co-factor in all tissues studied, and the assay consists in measuring the rate of inorganic phosphate released when the enzyme is saturated with calcium ions and ATP, with conditions such that the rate of phosphate release is proportional to time and to tissue concentration. The assay is carried out in a small volume to conserve ATP, using 10 x 50 mm. test tubes. However, the reaction may be carried out in a larger volume by keeping all the reactants at the same final concentration per unit volume. The assay is always carried out at two levels of tissue rather than in duplicate, since this provides a control on the technique.

TABLE XXVI

Reaction Mixture for ATP-ase Assay (37°C.)

Tube No.	1	2	3	4	5
	ml.	ml.	ml.	ml.	ml.
H_2O	0.20	0.10	0.45	0.25	0.30
0.5 M diethylbarbiturate pH 7.4	0.15	0.15	0.15	0.15	0.15
0.04 M $CaCl_2$	0.05	0.05	0.05	0.05	0.05
.013 M ATP, pH 7.4	0.15	0.15	--	--	0.15
1% rat liver homogenate (added 2 or 3 minutes after the tubes are placed in the thermostat)	0.10	0.20	--	0.20	--

The reaction is stopped by adding 0.1 ml. of 50% trichloracetic acid to each tube. The tubes are then centrifuged 10 minutes at 3000 r.p.m. and 0.3 ml. of the supernatants is carefully drawn off for analysis for inorganic phosphate. The reagent blank on the phosphate analysis is used for the I_0 readings, (100 setting on Evelyn colorimeter) and should include 0.3 ml. of the supernatant from tube No. 3. Tube No. 4 gives the inorganic phosphate from the tissue and No. 5 the spontaneous breakdown of the ATP. The assay is based on the amount of inorganic phosphate liberated from ATP per milligram of tissue in 15 minutes and is obtained from tubes 1 and 2 after applying the corrections obtained from tubes 3 and 4. The phosphate is determined by the Fiske-Subbarow method, as described on page 190 of this book.

The oxidative synthesis of phosphate bonds: It is possible to carry out oxidations over the cytochrome system and to use this energy for the esterification of inorganic phosphate using adenylic acid or ADP to accept the phosphate from the oxidized intermediates. However, ATP-ase assays as described in the preceding section show that any ATP formed will be split and inorganic phosphate will be returned to the medium, and the inorganic phosphate of the medium will not decrease unless the esterification can outpace the ATP-ase. If creatine or some other phosphate acceptor is added it is possible to tap off the ATP phosphorus and to minimize the amount of ATP available for dephosphorylation. Creatine seems to be ideal for this purpose. In addition, the action of ATP-ase is retarded by fluoride and magnesium ions, which can be added to the mixture, since fluoride does not inhibit the oxidations appreciably at the concentration used, and magnesium appears to be necessary for the phosphorylation. It must be emphasized that tissues are excised quickly and cooled on ice; they are homogenized rapidly and in the cold, and they are added to reaction mixtures held at zero degrees with all reactants present. As soon as the homogenate is added, the flasks are attached to manometers and placed in the 37° bath. They are equilibrated for 10 minutes; oxygen uptake is measured at 10 minute intervals. The flasks are taken from the manometers and placed in cracked ice; 2 ml. of 17.5% trichloracetic acid is used to stop the reaction. The flask contents are transferred to centrifuge tubes and centrifuged in the cold and kept in cracked ice when not in the centrifuge. The phosphorus distribution can be studied according to the methods of Chapter 15; when a volume of 3.0 ml. is used in the final tube containing reduced phosphomolybdate, we use 0.05 ml. and 0.10 ml. of the protein-free supernatant fluid for the analysis for inorganic P + phosphocreatine P. To determine "true" inorganic P, 2.0 ml. of protein-free supernatant fluid are brought to pH 8.3 (phenolphthalein) with 2N NaOH and 0.4 ml. of the calcium reagent is added. The precipitate is centrifuged down, drained by inversion and the excess supernatant fluid blotted with filter paper. The precipitate is suspended in water to give 1.8 ml. and dissolved by adding 0.2 ml. 2N HCl. The solution is then equivalent to the original, and "true" inorganic phosphate is determined in aliquots of 0.05 ml. and 0.10 ml. The phosphate content of the original flask is obtained by multiplying by 100 and by 100/2 respectively. The original P content is determined by adding the enzyme after the 2 ml. trichloracetic in one case. In these experiments ATP is added originally.

As it reacts with phosphocreatine, ADP and adenylic acid are made available to the oxidative P-donors.

It seems undesirable to have any more adenylic acid exposed to its deaminase than can be avoided. When the oxidative systems do not phosphorylate, the inorganic phosphate increases due to ATP breakdown, but when the system contains all the components, a net phosphate uptake is observed. This reaction is especially strong in kidney cortex homogenates but it also occurs in other tissues.

The reaction mixtures that are used for keto-acid oxidations are also useful for the study of oxidative phosphorylation and are superior to the mixtures that were used earlier. The results with water homogenates (Potter, 1945a, 1945b, and 1947) were inferior to those obtained in more recent studies with isotonic homogenates (Potter, LePage and Klug, 1948). It is possible to obtain phosphate uptake in the absence of fluoride, but much better uptake is observed when fluoride is present (Potter, 1947). Recent studies have shown that the phosphate uptake can be improved dramatically by using mitochondria separated from both nuclei and soluble enzymes, since the mitochondria appear to be the chief bearers of the enzymes involved in oxidative dephosphorylation, and the soluble enzymes include the adenylic deaminase (Potter and Schneider, to be published).

The reaction mixtures that are used for glycolysis are useful for the study of the anaerobic phosphorylation reactions (see LePage, 1948 and LePage and Schneider, 1948).

The preparation of systems which can maintain their energy reservoir (ATP) in the absence of fluoride will probably make it possible to study reactions hitherto considered "vital" and not open to study by cell-free techniques.

ARGINASE

A recent paper by Roberts (1948) on the determination of arginase in homogenates is mentioned because it appears to be a valuable contribution to methodology. Following earlier work with purified enzyme preparations, it was shown that the activation of arginase in homogenates required incubation with $MnCl_2$ for up to four hours to reach maximum values. The time for maximal activation was not the same in all tissues. With maximally activated homogenates the rate of reaction was linear, and proportional to the amount of tissue added.

GENERAL

The earlier practice of preparing extracts from tissue prior to the determination of various enzymes cannot be regarded as justifiable unless experiments are done to prove that interfering tissue elements are thereby removed. The use of the whole homogenate provides a point of reference for comparison with any fraction that may be derived therefrom, and if a homogenate is to be fractionated for assay purposes it would appear to be desirable to obtain nuclei, mitochondria, microsomes and soluble proteins in as physiological a condition as possible. Such attempts are beginning to be made (LePage and Schneider, 1948; Potter and Schneider, to be published; also see following chapter) and much further progress is anticipated.

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Chapter XII

METHODS FOR THE ISOLATION OF PARTICULATE COMPONENTS OF THE CELL

INTRODUCTION AND GENERAL PRINCIPLES

In his studies of tissue metabolism, the biochemist has directed his attention in two distinct ways, each of which has led him closer to an understanding of how cells and tissues function in terms of individual enzymatic reactions. The first studies dealt with the determination of metabolic reactions in cells and tissues and the quantitative measurement of the enzymes involved. More recently the biochemist has initiated studies of the distribution of enzymes within cells. This chapter is concerned with some of the methods employed in such studies.

Cells contain several discrete structures that can be seen under the microscope. The largest of these structures is the nucleus. One or more nucleoli can be seen within the nucleus and the chromosomes (chromatin material) may or may not be visible. In the cytoplasm are found the mitochondria, secretory granules, fat droplets, submicroscopic particles and soluble material. Of these the former three can be seen readily under the microscope while the submicroscopic particles are so small that they can be seen only as points of light in the dark field or ultramicroscope. The soluble materials are of course invisible.

Since cells are such complex structures it is rather obvious that the study of the intracellular distribution of enzymes must involve close collaboration between the cytologist and the biochemist. The cytologist must identify the structures isolated and the biochemist must study their enzymatic properties.

Since most tissues are composed of several different types of cells, it would appear that single cells and the particulate components isolated from single cells would be the ideal materials for the study of the intracellular distribution of enzymes. Linderstrøm-Lang, Holter and their associates (Linderstrøm-Lang, 1938) have in fact been able to make such studies on single cells. In general, however, the use of single cells appears to be distinctly limited both with respect to the types of cells and the enzymes that can be studied.

A method which permits full exploitation of current knowledge of enzymes was provided by the simultaneous development of the homogenate technique (Chapter 11) and the so-called cell fractionation technique. The homogenate technique provided methods for the quantitative assay of enzymatic activity in cell-free preparations of tissues (homogenates). In addition, it was found that the morphological and cytological properties of the particulate components of the cell were preserved when the homogenates were made in the proper medium and the homogenates could be fractionated by means of differential centrifugation into a nuclear fraction, a mitochondria fraction, a submicroscopic particle fraction, and a soluble fraction (Hogeboom, Schneider and Pallade, 1948).

In the study of the intracellular distribution of enzymes by the cell fractionation technique, several important principles must be followed. In the first place, the identity of the fractions must be established cytologically. Each fraction must be demonstrated to consist of a single particulate component the cytological and morphological properties of which closely parallel those of the particulate component in the living cell. Secondly, the enzymatic activity of each fraction must be determined, and it must be demonstrated that the sum of the activity of all the fractions equals the activity of the unfractionated homogenate. In enzyme assays, the possibility that inhibitors or activators are present in the homogenate must be considered. In such a case the distribution of enzyme and of inhibitor or activator may be entirely different with the result that the sum of the activities of the fractions will be greater or less than the activity of the homogenate. Several instances will be cited later in which this is true. In such cases it is necessary to measure the activities of the fractions in all permutations and com-

binations as well as separately in order to determine the localization of the inhibitor or activator, as well as to eliminate the possibility of denaturation during the isolation procedure.

When these requirements have been met the localization of enzyme in a cellular component is indicated in three ways: (a) a large percentage of the total activity of the homogenate is recovered in the fraction; (b) the specific activity of the fraction is several times as great as that of the homogenate; and (c) the specific activity of the fraction remains constant upon repeated sedimentation. These criteria have been met only for a very few enzymes (see Schneider, 1949). It is hoped that future research on the intracellular distribution of enzymes will adhere closely to these principles in order that the real significance of the association of enzymes with particulate components of the cell can be ascertained.

CYTOLOGICAL IDENTIFICATION OF PARTICULATE COMPONENTS OF THE CELL

The identification of particulate components of the cell requires the collaboration of a cytologist thoroughly versed in the morphology of living cells as well as in the classical staining properties of fixed cells. It is especially important that the identification be made on unfixed and unstained material supplemented by observations employing the usual staining methods. Only in this manner can the identification of cellular components be firmly established.

The study of cellular components in unfixed and unstained material has presented considerable difficulty in the past because of the slight differences between the index of refraction of the particulate components and the surrounding material. This difficulty can be eliminated largely by use of the dark field microscope and the recently developed phase microscope. The latter provides an especially easy method for the demonstration (in fresh preparations) of particulate components of the cell that are seen with great difficulty with the ordinary microscope.

The cytological identification of the submicroscopic particles is unsatisfactory. These particles are so small that they can not be resolved in the light microscope and as a result it is impossible to determine their morphological and cytological properties in the living cell. The electron microscope provides the only means for studying these particles, and this apparatus requires the use of dry preparations.

METHODS FOR THE COMPLETE FRACTIONATION OF RAT LIVER HOMOGENATES BY DIFFERENTIAL CENTRIFUGATION

Choice of Tissue for Centrifugal Fractionation: Since most mammalian tissues consist of several types of cells rather than of a single type as required for centrifugal fractionation, the choice of tissues is exceedingly important. Rat liver has proved to be an excellent tissue for centrifugal fractionation, mainly because it is composed largely of one type of cell. In addition, liver cells are easily broken and contain a variety of known enzymes. As a result liver tissue has been used in most of the studies on centrifugal fractionation. The fractionation methods described in this chapter have been devised for rat liver, and their application to other tissues may require some modification.

Centrifuges: In order to minimize autolytic processes during the centrifugal fractionation of tissues it is necessary to carry out all steps at as low a temperature as possible. For this reason the International Refrigerated Centrifuge PR-1 is the centrifuge of choice for most of the centrifugal procedures. This centrifuge permits the use of a variety of both horizontal and angle rotors in a refrigerated chamber which can be maintained at 32° F. ± 1° or at other selected temperatures. It is desirable to maintain the chamber at a temperature several degrees lower than required because the temperature of the rotor is usually several degrees above that of the surrounding air. With the multispeed attachment and rotor No. 295, forces as great as 30,000 g (middle of tube) can be obtained in this centrifuge. It is worthwhile to point out that the capacity of this rotor can be doubled by substituting 3/4" O.D. lusteroid tubes for the pyrex tubes and rubber jackets with which the rotor is supplied. If this is done it is necessary to put

2 ml. of water in each rotor hole as a cushion because the rotor holes are larger than the lusteroid tubes and also because the rotor holes have a conical bottom rather than a round one.

For centrifugal forces greater than those obtainable with the International centrifuge, the recently marketed Spinco centrifuge (manufactured by the Specialized Instruments Corp., Belmont, California) is recommended. This centrifuge is electrically driven and the rotor spins in an evacuated chamber. The latter prevents an appreciable temperature rise during the centrifugation. The maximum force obtainable with the preparative rotors supplied with this machine is 170,000 g and the largest volume that can be centrifuged is 460 ml. In addition, the centrifuge can be obtained with a refrigerated chamber and with the necessary optical equipment for analytical work.

Two formulae frequently used in centrifugal work merit inclusion in this discussion. The first deals with the determination of centrifugal force, and is given by the equation

$$(51) \quad F = \frac{W^2 r}{980} = \frac{S^2 r}{89500}$$

where F is the centrifugal force (in gravitational units, g) W is the angular velocity, r is the radial distance in cm. from the center of rotation, and S is the speed in revolutions per minute. Another useful formula is that given by Pickels (1943) for roughly estimating particle size or for approximating the time required to sediment particles of know physical properties. According to this equation

$$(52) \quad T = 54 \left(\frac{D-L}{D+L} \right) \left(\frac{N}{d^2 (\sigma - \rho) S^2} \right)$$

where T is the time in minutes, D is the radial distance in cm. from the boundary (or outer edge of the tube for complete sedimentation) to the axis of rotation, L is the radial distance in cm. from the meniscus to the axis of rotation, N is the viscosity of the fluid in poises, σ and ρ are the densities in grams per cubic centimeter of the particles and medium respectively, d is the average diameter of the particles in centimeters, and S is the rotational speed in R.P.M.

Methods of Preparation of Homogenates: The most satisfactory method available for preparing cell-free tissue suspensions involves the use of the Potter-Elvehjem glass homogenizer (Chapter 11). With this apparatus practically all the cells are broken and the nuclei and other sub-cellular components remain unbroken. Both the mortar and pestle and the Waring blendor give poor yields of broken cells as well as breakage of nuclei. Use of the latter also may be accompanied by excessive frothing and heating.

Selection of Media for Homogenization: The medium in which the cells are disrupted has a profound effect on both the cytological and biochemical properties of the particulate components of the cell. The morphological and biochemical properties of the mitochondria in different media have been studied most thoroughly and have therefore served as the basis for selection of media for centrifugal fractionation. Little is known about the morphological and biochemical properties of the nuclei, the nuclear components, and the submicroscopic particles in different media. The secretory granules are apparently broken when the liver cells are disrupted (see Hogeboom, Schneider and Pallade, 1948). When homogenates of rat liver are made in distilled water, the mitochondria become swollen and the cytochrome C present in them is not active as shown by the succinoxidase test in the absence of added cytochrome C (Chapter 11). Cytochrome C is also associated with the submicroscopic particles isolated from water homogenates of rat liver. It is apparently present in adsorbed form, because the cytochrome C is removed when these particles are washed in isotonic saline. In homogenates of rat liver made in isotonic saline or other salts, however, the mitochondria are strongly agglutinated, although the cytochrome C present in them is active in the succinoxidase system and the mitochondria are normal in size. As a result of this agglutination, the mitochondria sediment with the nuclei, and it is impossible to obtain an adequate separation of the two. The submicroscopic particles isolated from saline homogenates do not contain cytochrome C (see Schneider, Claude

and Hogeboom, 1948). Furthermore the mitochondria in saline or water homogenates do not stain vitally with Janus Green B, the generally accepted stain specific for mitochondria. Homogenates prepared in isotonic or hypertonic sucrose have proved to be the most suitable for centrifugal fractionation of tissues (Hogeboom, Schneider and Pallade, 1948). In these solutions the mitochondria are normal in size, stain with Janus Green B, and are not agglutinated. Moreover, in hypertonic sucrose many of the mitochondria are elongated, a morphological property of mitochondria previously observed only within living cells. Mitochondria isolated from hypertonic sucrose homogenates also differ biochemically from those isolated from isotonic sucrose homogenates as shown by the succinoxidase test in the absence of added cytochrome C. The latter differences have not been studied fully and do not permit a decision as to which concentration of sucrose is optimum for the isolation of mitochondria. For this reason and for others mentioned below the centrifugal fractionation of both isotonic and hypertonic sucrose homogenates will be described.

Centrifugal Fractionation of Hypertonic (0.88 M) Sucrose Homogenates of Rat Liver: (Hogeboom, Schneider and Pallade, 1948). Adult albino rats were fasted overnight to remove glycogen from the liver. The rats were killed by decapitation (if desired the livers may be perfused before removal from the animal). The livers were cooled in a beaker placed in an ice bath and were passed through a tissue masher fitted with a screen containing holes 1 mm. or smaller in size. This procedure removed a large part of the connective tissue framework of the liver. The liver pulp was then weighed and homogenized in 9 volumes of ice cold 0.88 M sucrose (Merck, reagent grade) in the all-glass apparatus of Potter and Elvehjem (see Chapter 11).

Ten ml. of homogenate were layered over 1.0 ml. of 0.88 M sucrose in a 15 ml. conical centrifuge tube and centrifuged for 10 min. at 600 g. Nuclei, unbroken liver cells, and red blood cells were sedimented in the lower sucrose layer with a minimum loss of mitochondria. The supernatant was withdrawn with a capillary pipette and recentrifuged twice more for 10 min. at 600 g. This procedure completed removal of nuclei and unbroken liver cells from the homogenate. The complete recovery of nuclei was indicated by the fact that the entire desoxypentose nucleic acid (a specific nuclear constituent) of the homogenate was recovered in this fraction (see Table XXVII) as well as by microscopic examination of the fractions. The three sediments were combined and resuspended in 0.88 M sucrose (Fraction N).

The supernatant remaining after the final sedimentation at 600 g was transferred to lusteroid tubes and was centrifuged for 20 min. at 24,000 g. The sediment consisted of three layers: (1) a small amount of brown material at the bottom of the tube, (2) an intermediate layer of firmly packed tan material, and (3) an upper layer of poorly sedimented pink-white material. The last consisted of submicroscopic particles and was removed when the supernatant, S_1, was withdrawn with a capillary pipette. The remainder of the sediment consisted of mitochondria and some submicroscopic material. The brown layer apparently contained mitochondria that were more firmly packed than in the bulkier tan layer. The sediment was resuspended in 0.88 M sucrose (this resuspension is conveniently made with a Lucite homogenizer machined to fit the lusteroid tubes) and was centrifuged for 20 min. at 24,000 g. The supernatant (Fraction W_m) contained some submicroscopic particles. This was indicated by the high pentose nucleic acid phosphorus to nitrogen ratio, a characteristic of the submicroscopic particles (Table XXVII). The sediment was resuspended in 0.88 M sucrose (Fraction M_w). This suspension was yellow-tan in color and showed pronounced birefringence of flow when stirred. The latter was considered to be caused by the rod-like shape of the majority of the isolated mitochondria in the suspension.

The supernatant, S_1, remaining after sedimentation of the mitochondria was transferred to another lusteroid centrifuge tube and centrifuged for 2 hours at 41,000 g to sediment the submicroscopic particles. The supernatant, S_2, was removed with a capillary pipette. The sediment was transparent and reddish-brown in color. It was resuspended in 0.88 M sucrose (Lucite homogenizer) and recentrifuged 2 hours at 41,000 g. The supernatant from this centrifugation (Fraction W_p) was withdrawn and the sedimented submicroscopic particles were resuspended in 0.88 M sucrose (Fraction P_w). The latter fraction also exhibited strong birefringence of flow. Calculations using equation (52) above indicated that the force employed was only sufficient to sediment particles larger than 100 mμ. Thus the complete separation of submicroscopic material was probably not effected by this

centrifugation. (A more complete separation of submicroscopic particles from 0.88 M sucrose solutions has recently been accomplished by centrifuging for 1 hour at 170,000 g in the Spinco centrifuge.)

TABLE XXVII

Distribution of Nitrogen, Nucleic Acids, and Succinoxidase Activity in Fractions Isolated from Homogenates of Rat Liver in 0.88 M Sucrose (from Hogeboom, Schneider and Pallade, 1948)*

	Nitrogen	Succinoxidase		Pentose nucleic acid phosphorus (PNA-P)		Desoxypentose nucleic acid phosphorus (DNA-P)	
	Total (mg.)	Total (mm^3 O_2 per hr)	$Qo_2(N)$ (µl. O_2 per hr. per mg N)	Total (γ)	$\frac{\gamma \text{ PNA-P}}{\text{mg. N}}$	Total (γ)	$\frac{\gamma \text{ DNA-P}}{\text{mg. N}}$
H (homogenate)	33.6	25800	770	762	22.7	260	7.7
N	7.58	4750	630	144	18.9	258	34.1
M_w	8.75	18600	2120	136	15.5		
W_m	2.20	500	200	90.6	41.1		
P_w	2.98	< 250	< 100	174	58.0		
W_p	1.28			43.8	34.0		
S_2	10.5	< 250	< 30	161	15.3		
M (mitochondria sedimented once)	10.95	17300	1580	240	21.9		
M_{w1} (mitochondria sedimented twice)	8.45	16900	2010	119	14.1		
M_{w2} (mitochondria sedimented three times)	7.65	16300	2120	95	12.4		

*Each value in the table represents the amount found in or derived from 10 ml. of a 10 per cent liver homogenate.

The results of typical fractionations in 0.88 M sucrose are presented in Table XXVII and illustrate some of the fundamental principles outlined in the introductory paragraphs. In these experiments the distribution of succinoxidase activity in rat liver fractions was determined. The validity of the succinoxidase assay was indicated by the fact that about 94 per cent of the activity of the homogenate was recovered in the six fractions obtained.

The finding of major interest was that the succinoxidase system of the liver cell was apparently localized in the mitochondria. This was strongly indicated by the fact that the major portion of the total succinoxidase activity of the homogenate was recovered in the mitochondria fraction and that the specific activity of the latter was several times as great as that of the homogenate. That the succinoxidase activity was firmly bound to the mitochondria was evident, for only slight losses in activity occurred upon repeated sedimentation (see last three lines, Table XXVII).

Centrifugal Fractionation of Isotonic (0.25 M) Sucrose Homogenates of Rat Liver: (Schneider, 1948). Isotonic sucrose (0.25 M) possesses several advantages over hypertonic sucrose (0.88 M) as a medium for the centrifugal fractionation of rat liver. The activity of some complex enzyme systems, such as the system that oxidizes octanoic acid (octanoxidase), is much greater when the homogenates are made in isotonic sucrose than when made in hypertonic sucrose. Why this should be true is not at all clear, because other enzymes, such as those of the succinoxidase system, are only slightly affected by hypertonic sucrose. A further advantage in the use of isotonic sucrose is that greatly decreased centrifugal forces can be employed in the centrifugal fractionation, for isotonic sucrose has a much lower viscosity and specific gravity than hypertonic sucrose. As a result, the entire fractionation in isotonic sucrose can be made in the International centrifuge.

The preparation of the homogenate was similar to that described in preceding paragraphs for hypertonic sucrose; however, the fractionation differed from that with hypertonic sucrose. Ten ml. of a rat liver homogenate in 0.25 M sucrose was centrifuged for 10 min. at 600 g to sediment nuclei, unbroken liver cells, and red blood cells. The sediment was washed twice by rehomogenizing each time with 2.5 ml. of isotonic sucrose and centrifuging for 10 min. at 600 g. This treatment served to break up any intact liver cells as well as to remove any mitochondria that may have sedimented during the first centrifugation. The sediment remaining after the final centrifugation was resuspended in 0.25 M sucrose and labeled the nuclear fraction N_W. This fraction contained all the nuclei of the homogenate as shown both by microscopic examination and analysis for desoxypentose nucleic acid. The nuclei were agglutinated in large clumps and the fraction contained a few free mitochondria and intact liver cells in addition to the nuclei and red blood cells.

The supernatants obtained from the nuclear fraction were combined and centrifuged for 10 min. at 8,500 g. in a lusteroid tube to sediment the mitochondria. The appearance of the sediment was similar to that described for hypertonic sucrose. The sedimented mitochondria were washed twice by resuspending (Lucite homogenizer) in 2.5 ml. of 0.25 M sucrose and recentrifuging at 8,500 g. The final sediment was resuspended in isotonic sucrose and labeled the mitochondria fraction, M_W. Microscopic examination showed that this fraction contained only mitochondria. Absence of submicroscopic particles was further indicated by the low pentose nucleic acid phosphorus to nitrogen ratio.

The supernatants from the mitochondria fraction were combined and centrifuged 1 hour at 18,000 g in lusteroid tubes to sediment the submicroscopic particles. The sediment was transparent and red-brown in color as was noted in 0.88 M sucrose. The sediment was washed once by resuspending in 2.5 ml. of 0.25 M sucrose (Lucite homogenizer) and resedimenting at 18,000 g. The final sediment was resuspended in 0.25 M sucrose and labeled the submicroscopic particle fraction, P_W. Microscopic examination (dark field) revealed a large number of particles which could not be resolved because of their small size but which were visible as pin points of light in rapid Brownian movement. The supernatants from the submicroscopic particles were combined to form the supernatant fraction, S_2.

Table XXVIII presents the results of the assay of liver fractions for three complex enzyme systems: the octanoxidase and oxalacetic oxidase systems (the enzyme systems that oxidize octanoic and oxalacetic acid, respectively) and the glycolytic system. In each case the sum of the activities of the four fractions (nuclear, N_W; mitochondria, M_W; submicroscopic particles, P_W; and supernatant, S_2) failed to equal that of the homogenate. This was especially true of the oxalacetic oxidase and glycolytic activities. However, when the fractions were recombined in various permutations and combinations and the activities determined, it was possible to obtain the full activity of the homogenate. Thus the possibility (mentioned in earlier paragraphs) that inhibitors or stimulators of enzymatic activity could be present in the homogenate and be distributed in various fractions was realized. In the case of the octanoxidase and oxalacetic oxidase systems, the main activ-

TABLE XXVIII

Distribution of Octanoxidase and Oxalacetic Oxidase Activity in Rat Liver Fractions and of Glycolytic Activity in Rabbit Liver Fractions (from Schneider, 1948; Schneider and Potter, 1949; LePage and Schneider, 1948).

	Octanoxidase Activity		Oxalacetic oxidase Activity		Glycolytic Activity	
	Total*	% of H	Total*	% of H	Total**	% of H
H (homogenate)	4980	(100)	6180	(100)	313	(100)
N_w	138	2.8	648	10.5	39.5	12.6
M_w	4020	80.7	2750	44.5	0	0
P_w	0	0	0	0	8.5	2.7
S_2	0	0	31	0.5	165	52.7
$N_w + M_w$	4740	95.0	--	--	42	13.4
$N_w + P_w$	66	1.3	--	--	87	27.8
$N_w + S_2$	--	--	--	--	238	76.0
$M_w + P_w$	4740	95.0	5040	81.5	27.5	8.8
$M_w + S_2$	--	--	3830	62.0	215	68.6
$P_w + S_2$	--	--	53	0.9	297	94.9
$N_w + M_w + P_w$	5040	101	--	--	--	--
$M_w + P_w + S_2$	--	--	6060	98.0	--	--
$N_w + M_w + P_w + S_2$	--	--	--	--	328	104.8

*Cu. mm. O_2 per hr. per 10 ml. of 10 per cent homogenate or its equivalent.
**Micromoles lactic acid produced per hr. per 10 ml. of 10 per cent homogenate or its equivalent.

ity appeared to be associated with the mitochondria, whereas glycolytic activity was associated mainly with the supernatant. The fractions that contained the major activity of the homogenate were stimulated by other fractions that had little or no activity by themselves. Thus the octanoxidase activity of the mitochondria was increased by the submicroscopic particles, and the oxalacetic oxidase activity of the mitochondria was greatly augmented by the submicroscopic particles or the supernatant. The glycolytic activity of the supernatant was greatly increased by the addition of the mitochondria or the submicroscopic particles. Although these stimulatory effects are not fully understood, further investigation should provide useful and interesting information concerning the mechanisms by which the actions of the components of the cell are integrated to produce the enzymatic activity of the entire cell.

METHODS FOR THE ISOLATION OF SINGLE PARTICULATE COMPONENTS OF THE CELL

Several methods are available for the isolation of single particulate components of the cell. These will not be described in detail because the author has had little or no experience in their application. It is necessary to caution again that if it is decided to isolate individual particulate components of the cell (rather than to attempt the complete fractionation described above) that all fractions obtained be assayed for enzymatic activity and not merely the fraction in which one is interested.

Isolation of Nuclei: Dounce (1943) has published a method for the isolation of nuclei from rat liver and has studied a number of enzymes associated with them. The method consisted of differential centrifugation of rat liver homogenates in dilute citric acid (final pH 6.0-6.2). The nuclei were not isolated quantitatively and the enzymatic activity of the isolated nuclei was compared with that of the whole liver. No attempt was made to determine the activity of other fractions obtained. With the exception of alkaline phosphatase the enzyme concentration in the nucleus was considerably less than in the whole tissue. The significance of the association of such low concentrations of enzymes with the nuclei is doubtful.

Another method (Behrens, 1939) for the isolation of nuclei from animal tissues involves the lyophilization of the tissue followed by grinding and suspension in a mixture of organic solvents of selected specific gravity. The suspension is centrifuged to obtain nuclear and cytoplasmic concentrates. Apparently the specific gravity of the nuclei and of the cytoplasm is sufficiently different to permit a separation. This method was used to study the distribution of arginase and lipase in rat liver but the results are not entirely clear because the data were not complete. Improved methods of lyophilization now available may warrant further study of this method.

Methods for the isolation of nuclei from bird erythrocytes, spermatazoa, and egg cells have been summarized in the paper of Dounce (1943). Few enzymatic studies have been made with these nuclei preparations.

Isolation of Chromosomes: The isolation of chromosomes was first reported by Claude and Potter (1943). This was accomplished by grinding the tissue with sand to break the nuclear membrane and thus release the nuclear contents. The chromosomes were then obtained by differential centrifugation. While working with Dr. Claude, the author learned that the sand must be extremely fine to break the nuclear membrane and it appeared that the diameter of the sand (or quartz) particles needed to be smaller than that of the nuclei.

Another method for the isolation of chromosomes was described by Mirsky and Ris (1947). This method was similar to that of Claude and Potter with the exception that the nuclear membranes were broken by the use of the Waring blendor rather than by grinding with sand.

Enzyme studies on the isolated chromosomes have not been reported.

Isolation of Chloroplasts: Chloroplasts were isolated from leaves by a method described by Granick (1938). The leaf cells were disrupted by grinding with sand (the Potter-Elvehjem homogenizer would seem to be a more suitable apparatus) and the chloroplasts were isolated by differential centrifugation. The chloroplasts present in each fraction were quantitated by extracting the chlorophyll and measuring the color density in the Duboscq colorimeter. It is of interest to note that Granick found that 0.5 M glucose or sucrose solutions were the best media for the preservation and isolation of chloroplasts. Salt solutions caused coagulation and granulation. These results are strikingly similar to those recently obtained with rat liver (Hogeboom, Schneider and Pallade, 1948).

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Chapter XIII

CHEMICAL METHODS

Manometric methods find their widest use in the study of tissue metabolism. However tissue metabolism consists of many reactions not involving gaseous change, or reactions measurable by gas exchange are often associated with other reactions not so measurable. It is therefore very convenient to have available chemical methods to determine the disappearance of substrates, the appearance of products, or the change in one or more of the substances in the medium in which the tissue is suspended.

Since the manometric methods described are capable of measuring very small changes, chemical methods employed must be capable of measuring changes of the order of 5 to 10 micrograms. Their total range usually must be from 0 to 100 micrograms. The small quantities involved exclude all but the simplest fractionation procedures, so that any methods employed must be specific. In addition they usually must be specific enough to measure small quantities of the compound involved without interference by other related materials which may be present in large quantities. Means of achieving specificity in micro-methods are described below.

For practical purposes colorimetric methods are the only ones available. The principles underlying these methods are described below in the section on colorimetry. Clinical handbooks, manuals of methods, etc., abound in colorimetric methods. An examination of the recent literature will often disclose methods suitable for use with the respirometer.

It is not our purpose to describe a large number of methods. We are concerned only with basic methods which would probably be used frequently by one working with manometric techniques.

PREPARATION OF TISSUE FOR ANALYSIS

The usual procedure for the analysis of the contents of respirometer flasks involves stopping the reactions at the desired point. Usually this is done by adding, at the appropriate time, sufficient trichloracetic acid to yield 5-10% (by weight) final concentration of the acid. A solution of trichloracetic acid containing 100 g. of acid per 100 ml. is readily prepared and convenient for such additions. Addition of 0.33 ml. of the solution from the sidearm of a flask containing 3 ml. of solution will serve to stop the reactions, precipitate the proteins and frequently will extract materials from the cells. The contents of the flask are removed, centrifuged, and the clear supernatant fluid is used for analysis.

GENERAL PRINCIPLES

As indicated above, specificity is one of the chief considerations in the choice of a colorimetric method. This requirement is satisfied either by (1) finding a reaction essentially specific for the material to be measured or (2) by separating the component to be measured from interfering materials. An example of an inherently specific method is the colorimetric method for phosphorus (conditions are chosen to minimize interference from arsenic and silica). As most methods are less specific than this, it often is necessary to resort to fractionation. In the following section methods designed to purify materials on a micro scale by distillation, precipitation, extraction and adsorption are described.

Distillation: If the material to be measured is volatile (ammonia, ethanol, diacetyl, etc.), if it can be converted into a volatile material (as an ester to the free acid and alcohol), or if substances which interfere with the color reaction (or other method of estimation) can be removed as volatile materials, a process of distillation is indicated. Small stills employing either direct distillation over a flame or employing steam (the usual micro Kjeldahl still) are capable of handling quantities of 10-100 µg. However,

there are certain other ingenious devices which permit distillation from the frozen state or distillation in vacuo which are particularly convenient for manometric work. Some of these are mentioned below, and it is assumed that any specific method will require modification for the particular circumstances under which one is working. In vacuo distillation from the frozen state avoids heat decomposition and may be conducted essentially as follows in a device described by Grant (1946): The substance to be distilled is placed, by means of a curved pipette in compartment A, Fig. 52, which is then immersed in a dry ice-acetone bath until frozen solid. C is then attached to a rotary-seal oil pump and the system is thoroughly evacuated and closed off under vacuum by stopcock D. The B section of the tube is then immersed in the dry ice-acetone bath while portion A remains outside of it. In the course of 4 or 5 hours all the volatile material in A distills over (usually without melting) and condenses in B. It is important in this method that A be taken completely to dryness. Modifications of this procedure are obvious. Large size Thunberg tubes may be used with the stopper serving as A and the tube as B. The small size (15 ml.) tube has too small an opening to permit adequate distillation. A relatively volatile material which can be distilled into a substance to yield a non-volatile derivative (ammonia into acid, an aldehyde into hydrazine, etc.) may be distilled directly in a Warburg flask. The trapping agent is put in the sidearm. The flask contents are frozen and while frozen are evacuated by attaching to an inner standard taper joint equipped with a stopcock and connected to a rotary-seal oil pump. After evacuation the system is allowed to stand either at room temperature or in a warm water bath or incubator until distillation is complete (2-3 hours or overnight).

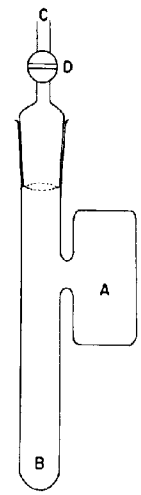

FIG. 52

Tube for vacuum distillation from the frozen state.

For similar determinations a Conway diffusion dish may be employed (Fig. 53). In the determination of ammonia, for example, the sample is placed in A and standard H_2SO_4 is placed in B. The glass plate is put on, the sample mixed with alkali (placed at another spot in trough A), and incubated 6-8 hours at 37° C. The ammonia is trapped in the H_2SO_4 and is determined by titration or Nesslerization. Similar methods of distillation are employed for other volatile materials. Examples of the application of the method are given by Conway (1940), Borsook and Dubnoff (1939), Werch (1941), Winnick (1941, 1942), Warner (1942), and Malley, et al. (1943). Ceramic Conway dishes are obtainable from Arthur H. Thomas Company and dishes made from plastic and glass are sold by Scientific Service, Inc., Berkeley 3, California.

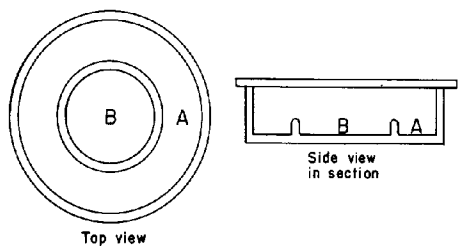

FIG. 53

Top and side views of Conway diffusion dish.

Precipitation: If the compound to be measured will form very insoluble precipitates that can be reacted to produce a color, the compound can be separated and estimated in this manner. The handling of such small precipitates is a difficult task, but is usually eased by adding an inert material such as barium sulfate or diatomaceous earth on which the smaller precipitate may be adsorbed. Such a procedure has been employed, for example, by Grant (1947) in the determination of formic acid. Further examples of the use of precipitation as a tool in colorimetric analysis are given in Chapter 15 on the determination of phosphorylated intermediates.

Extraction: The material to be estimated may be extracted from the reaction mixture (e.g., ether extraction is used in the determination of succinic acid, page 166). When the proper solvents are chosen, an extraction, for example, from acid solution with an immiscible solvent followed by reextraction of this solvent with an alkaline solution may give good specificity to a relatively non-specific starting colorimetric reaction. Extraction does not have to be complete if the percentage extraction is reproducible under

standardized conditions. Rather simple extraction procedures may be employed; for example, one may determine keto acids by reacting them under acid conditions with 2,4-dinitrophenylhydrazine, extracting the mixture by shaking with an equal volume of toluene, and then adding a specified portion of the toluene to alcoholic KOH. Toluene extracts primarily the derivative of pyruvic acid, ethyl acetate extracts more of the dicarboxylic acids; use of these and other solvents may provide a simple analytical separation of keto acids. It is apparent that extractions need not necessarily be elaborate and laborious affairs and that they have a much wider application than is usually considered.

The simple, economical and compact apparatus illustrated in Fig. 54 may be employed for a variety of extractions. The basic unit is a 25 x 200 mm. Pyrex tube connected to a reflux condenser; the tube is indented to hold the sample reservoir above the boiling chamber. By placing an alundum or paper extraction thimble containing a solid above the indents a continuous drip extraction may be performed (Fig. 54,A). A Soxhlet thimble also furnishes a device for the extraction of a solid sample (Fig. 54,B). For extraction from a liquid by a non-miscible liquid of lower specific gravity the arrangement shown in Fig. 54,C is employed; this is much more compact than the usual Kutscher-Steudel extractor with a sidetube. The arrangement shown in Fig. 54,D permits extraction from a liquid by another liquid of greater specific gravity (the lighter liquid is added last to the inner tube which is open at both ends). By inserting a set of stainless steel or block tin tubes in a common water bath to serve as condensers a compact extraction unit may be built; 24 extractors should occupy only about 1 to 1.5 square feet of desk space. In using this apparatus concentrations normally are adjusted so that the entire extract in one tube is analyzed as one sample; this avoids the necessity for preparing aliquots after extraction.

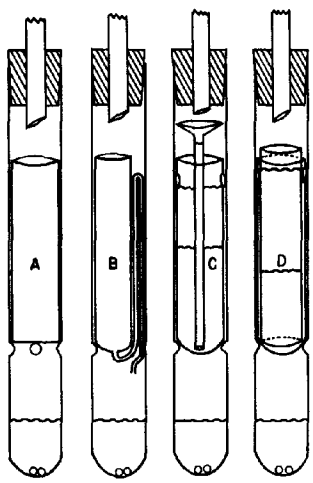

FIG. 54

Various forms of extraction apparatus. See text for description.

Adsorption and Chromatography: If one is able to find an adsorbent relatively specific for either the compound to be estimated or for the interfering substances, an adsorption process may be introduced to enhance specificity. An example is the colorimetric method for thiamine (Hennessy and Cerecedo, 1939). In addition there are the very valuable methods of partition chromatography on paper and on columns, which in many cases are adaptable to quantitative use (Martin and Synge, 1945; Williams and Kirby, 1948; Isherwood, 1946).

W. W. Umbreit

COLORIMETRY

Colorimetric methods of quantitative analysis are advantageous in many instances because of their speed, simplicity, precision, and a range extending downward beyond that of volumetric and gravimetric methods. Colorimetric methods are suited to the determination of the concentration of substances which are colored, or of uncolored substances which are capable of reacting quantitatively with suitable reagents to produce colored substances or which affect the equilibrium between color forms of indicator substances. These methods employ either visual or photoelectric colorimeters to determine the intensity of light transmitted by a colored solution compared to that transmitted by a reference standard.

Regardless of the colorimeter employed, the determination depends on the application of physical laws related to the transmission of light by homogeneous media, in this case colored solutions. When monochromatic light is used, successive layers of equal thickness of a colored solution absorb equal fractions of the incident light (Lambert's law), which means that the fraction of light absorbed increases in direct proportion to the logarithm of increase in thickness of the solution layer; also, the light absorbed by a layer of solution is directly proportional to the concentration of the colored substance (Beer's

law). These laws, usually designated as the Lambert-Beer law, are most conveniently represented for colorimetry in the form of the following equation:

$$(53)\quad I/I_o = e^{-kc\ell}$$

which may also be written:

$$(54)\quad -\log I/I_o = k'c\ell$$

where I_o is the intensity of the incident light, and I the intensity of the light transmitted by ℓ centimeters of a solution of c concentration (e.g., grams per 100 ml., or moles per liter) of colored solute having the characteristic absorption coefficient k, or specific extinction k'. Since this equation holds strictly only when monochromatic light is employed, it is to be expected that the greatest deviations occur when white light is used. The situation is considerably improved and in many instances completely rectified for all practical purposes by using a filter to restrict the light to a band of wave lengths, or color, complementary to the color of the colored substance being determined.

Visual Colorimetry: The ratio I/I_o, which need not involve actual values for I and I_o, represents the fraction of light transmitted by a colored solution. In direct-comparison colorimetry when a match of the transmission of an unknown solution of thickness ℓ is made with one of a series of standards (each of thickness ℓ, and c varying) of the same colored solute,

$$I/I_{o(\text{unknown})} = I/I_{o(\text{standard})}$$

and the concentration of the colored solute is the same in each.

Perhaps the most familiar application of the Lambert-Beer law in visual colorimetry occurs in connection with the use of colorimeters of the Duboscq type where the thickness of the layers of two solutions differing only in respect to the concentration of a colored solute are adjusted until a color match is obtained. Then,

$$-\log(I/I_o)_1 = -\log(I/I_o)_2 = k'c_1\ell_1 = k'c_2\ell_2$$

whence,

$$c_1\ell_1 = c_2\ell_2$$

If the concentration of the colored solute of one of the solutions is known (the standard), the concentration of the colored solute in the other solution (the sample) may be calculated readily. Best results are obtained when the concentrations of the standard and sample are of the same order of magnitude. In any case where a method is being instituted one should determine that Beer's law holds sufficiently over the working range of concentrations for the precision desired.

Photoelectric Colorimetry: At present it appears that visual colorimeters will be almost, if not completely, replaced by photoelectric colorimeters. This would seem to be a natural development in colorimetry following the development of sensitive, rugged, electrical devices for measuring light intensity. With colorimeters employing such devices the accuracy of a determination is not dependent on the visual acuity of the operator. Usually sensitive photocells are used to measure the light transmitted; it is necessary to express this in arbitrary units only. These photocells extend the range of colorimetric analysis to include very low concentrations of a colored substance. They allow more effective use of filters to obtain light that is selectively absorbed by a particular colored substance. This not only increases the accuracy of a determination, but also may allow one colored substance to be determined in the presence of others, or two or more colored substances to be determined in the same solution. When more than one colored substance is present, they can be determined best when they possess absorption bands in widely different parts of the spectrum. Then by successively using a light filter transmitting light absorbed only by one of the colored substances present, each substance can be determined in turn.

Photoelectric colorimeters are usually designed such that the layer of the colored solution is constant. This is assured by the use of absorption cells of various types that can be accurately aligned in the light beam so that the light traverses the same thickness of solution each time. Because of the inconvenience of preparing standards, and because a more complicated light system and electrical circuit is entailed in comparator type instruments, most modern photoelectric colorimeters are designed to determine directly the fraction of light transmitted by the colored solute in the sample. The value obtained is then used to interpolate the result graphically, i.e., the concentration of the sample is read from a calibration curve. The calibration curve is obtained by plotting the fraction of light transmitted against various known concentrations of the colored substance under the same conditions used for the sample. These conditions include the use of: the same wavelength band of light; the same concentration of reagents; the same solvent; and the same absorption cell, or matched absorption cells. Due consideration must be given to the timing of operations in those instances where a period of time is required for the maximum color to develop after the reagents have been added to the sample, or where the color is stable only over a certain period of time. Ordinarily, temperature is not critical since the determinations are carried out over a rather narrow range of room temperatures; however, it may become an important factor if the temperature variation is sufficient to cause changes in ionization, dissociation, or a shift in equilibrium involving the colored substance. When heat is used to develop a colored substance, the solution is cooled to room temperature before its transmission is measured.

It is evident from a consideration of Equation 53 or 54 that if ℓ is held constant the concentration of a colored substance is inversely proportional to the light transmitted, i.e., a straight line should be obtained if $\log I_0/I$ is plotted against concentration. However, in actual practice this may not be the case; rather the line may be curved, or it may be essentially straight over only a portion of the concentration range. These deviations may result from the use of light which is not sufficiently monochromatic, the presence of interfering colored substances, or changes in the absorbing material itself due to different degrees of ionization, dissociation, or combination with the solvent or reagents present as its concentration is changed. The deviation is corrected for if one prepares a standard curve and uses it to interpolate the data obtained. It is particularly desirable to determine whether or not a straight line is obtained, i.e., whether or not Beer's law holds for the set of experimental conditions and over the range of concentration of the colored substance being determined. If the line is straight, any number of final results may be obtained more readily and perhaps more accurately by calculation using a suitable calibration factor than by interpolation from a standard curve.

A calibration factor for use in calculating the concentration of a colored substance from the "reading" obtained with a photoelectric colorimeter may be obtained readily. In the two general procedures outlined below, where two types of colorimeters are employed, it should be understood that we do not wish to imply that the colorimeters named are necessarily the most satisfactory. It so happens that we have had occasion to use the two instruments, but the choice of a colorimeter is left to the individual investigator.

For photoelectric colorimeters employing a galvanometer connected directly with a photocell to measure the light intensity, e.g., the Evelyn photoelectric colorimeter (cf. Bulletin 460 of the Rubicon Company, 29 N. Sixth Street, Philadelphia), the light intensity is regulated to give a constant galvanometer deflection of 100 scale divisions when a blank (the solvent only, or a test mixture containing a quantity of pure water equal to that of the sample to be used treated with the same reagents, etc.) is inserted in the light beam. The blank is then replaced with a standard and the galvanometer reading, G, noted. The calibration factor, K, is then,

$$K = 1/c\ (\log 100 - \log G)$$

$$= 1/c\ (2 - \log G)$$

Then in using the calibration factor,

$$c = 1/K\ (2 - \log G)$$

The actual calculations are simplified by the use of a 2-log G table supplied with the instrument.

In the case of colorimeters which employ a potentiometer circuit to balance the output of two photoelectric cells, e.g., the Klett-Summerson photoelectric colorimeter (cf. General Directions for this instrument, Klett Manufacturing Company, New York), the galvanometer is first adjusted to zero with the blank in the light beam by varying a suitable resistance; with the standard replacing the blank the galvanometer is readjusted to zero by adjusting the potentiometer. Since the potentiometer scale is logarithmic the scale reading, R, is directly proportional to the concentration of the standard. Then,

$$K = 1/R(c)$$

and in using the calibration factor,

$$c = KR$$

Thus in either case outlined above the calculations are no more difficult than those necessary when a visual comparator-type colorimeter is employed.

Further information on colorimetry and colorimeters may be found in books by Gibb (1942) and Snell and Snell (1948). Brochures published by the manufacturers of colorimeters are generally replete with information on the care of these instruments, details of operation and specific applications.

J. F. Stauffer

METHOD FOR NITROGEN

Total Nitrogen: Johnson (1941) has described the following micro method for determining nitrogen. A sample containing 10 to 40 micrograms of nitrogen is pipetted into an 18 x 150 mm. Pyrex test tube (the tubes are matched for colorimetric analysis), 1 ml. of 2 N H_2SO_4 containing 0.2 g. per liter of $CuSeO_3$ (or an equivalent mixture of $CuSO_4$ and Na_2SeO_3) is added, and the tube is covered with a loose glass cap. The contents of the tube are then digested overnight in a digestion rack kept in an oven. The electrical elements serving as the heat source for the oven are located at the base of the tubes; they raise the temperature of the oven to 100-115° C. which insures rapid evaporation of water, but the temperature at the base of the tubes is much higher. The heating is such that after the water has evaporated, the H_2SO_4 condenses within 3 cm. of the bottom of the tube. To the tube, after digestion, are added in order 2 ml. of water, 2 ml. of color reagent, and 3 ml. of 2 N NaOH. (The color reagent contains per liter, 4 g. of KI, 4 g. of HgI_2, and 1.75 g. of gum ghatti. Dissolve the KI plus HgI_2 in 25 ml. water. Select light colored pieces of gum ghatti to reduce the blank, grind them in a mortar, drop the powder into 750 ml. of boiling water and reflux until dissolved. Add the KI and HgI_2 solution to the gum ghatti solution, dilute to 1 liter and filter. Replace the filter paper periodically to speed filtration.) After standing 15 minutes, the tube is placed in a photoelectric colorimeter, and a reading taken. A 490 mμ filter is used. Blanks and nitrogen standards are always run with each series of samples. The extinction coefficient is proportional to the quantity of nitrogen for samples containing less than 45 micrograms of nitrogen. Known samples can be recovered with an error of 3% or less. When this method is used with samples having a low percentage of nitrogen, 2 ml. of digestion reagent may be used. A drop of nitrogen-free 30% H_2O_2 may be added if digestion proves difficult.

Ammonia: Place an aliquot containing less than 40 micrograms of ammonia nitrogen in an 18 x 150 mm. test tube and adjust the volume to 2 ml. Add 2 ml. of color reagent and then 3 ml. of 2 N NaOH, and proceed with the determination as described under total nitrogen.

METHOD FOR GLUCOSE AND OTHER SUGARS

Details of detection and quantitative estimation of a number of sugars are given by Bates (1942) and by Gurin and Hood (1939, 1941). Methods for pentoses (ribose, arabinose, xylose) and for fructose are given in Chapter 15.

Glucose may be determined by the method of Folin and Malmros (1929). The sample is made to 4 ml. in a colorimeter tube, 2 ml. of 0.40% $K_3Fe(CN)_6$ and 1 ml. of carbonate-cyanide mixture are added. The sample is mixed, heated 8 minutes in a boiling water bath, cooled 1-2 minutes, and 5 ml. ferric iron solution is added to produce the color. The volume is made to 25 ml. with distilled water, and the color is read using a 520 mμ filter.

Range: 10-100 micrograms glucose equivalents (this method actually measures "reducing value" rather than glucose as such, and results are reported under the term "reducing value" in Chapter 15). Precision: ± 0.4 microgram.

Reagents: Carbonate-cyanide: Dissolve 8 grams anhydrous Na_2CO_3 in 40-50 ml. water, and add 15 ml. freshly prepared 1% NaCN. Dilute to 500 ml. Stable for long periods. Ferric iron: Soak 20 grams gum ghatti in one liter of water for 24 hours. The gum is suspended in the water in a cheesecloth bag. Add a mixture of 5 grams anhydrous $Fe_2(SO_4)_3$, 75 ml. 85% H_3PO_4 and 100 ml. water. After mixing add slowly about 15 ml. of 1% $KMnO_4$ to destroy reducing materials present in the gum ghatti and allow the solution to stand for a few days. Stable for long periods.

Micro modification: Park and Johnson (1949) have modified the method to make it suitable for the analysis of smaller amounts of glucose. The procedure follows: Dilute the sample to 1 ml., add 1 ml. carbonate-cyanide mixture and 1 ml. $K_3Fe(CN)_6$ solution. Mix. Heat 15 minutes in a boiling water bath. Cool. Add 5 ml. ferric iron solution. After 15 minutes, read in a colorimeter with a 690 mμ filter. Range: 1-9 micrograms. Precision: ± 0.2 micrograms.

Reagents for micro modification: Ferricyanide solution: 0.5 g. $K_3Fe(CN)_6$ per liter; store in a brown bottle. Carbonate-cyanide: 5.3 g. Na_2CO_3 plus 0.65 g. KCN per liter. Ferric iron: 1.5 g. $FeNH_4(SO_4)_2 \cdot 12H_2O$ plus 50 ml. 1 N. H_2SO_4 plus 1 g. Duponol (ME dry); diluted to 1 liter.

METHODS FOR PHOSPHORUS

Phosphorus may be determined by methods described in Chapter 15. If a slightly longer range is desired the following procedure may be employed:

The sample is mixed in a colorimeter tube with 2 ml. 2.5% ammonium molybdate made up in 5 N H_2SO_4, and the sample is diluted to about 20 ml. One ml. reducing reagent is added, and the sample is diluted to 25 ml. Range: 10-100 micrograms phosphate-phosphorus. Precision: ± 0.3 microgram. Micromodifications are easily devised by a proportionate reduction of all reagents.

METHOD FOR LACTIC ACID

Lactic acid may be determined by the method described in Chapter 15, page 192.

W. W. Umbreit

METHOD FOR PYRUVIC ACID

Pyruvic acid is readily determined in biological materials by the method of Lu (1939) as modified by Bueding and Wortis (1940) and Elgart and Nelson (1941). When pyruvic acid is to be determined in blood it is essential that it be stabilized immediately by the addition of iodoacetate to a final concentration of 0.2%. The iodoacetate prevents the loss of blood pyruvate but care should be taken to deproteinize the blood as soon as possible in order to prevent an increase in pyruvate. Suspensions of cells or tissue homogenates which have been used in manometric experiments may be pipetted directly from the respirometer flask into trichloracetic acid.

Reagents: Solutions are given as grams of solute per 100 ml. final volume of solution. (1) 25% solution of iodoacetic acid in water adjusted to pH 7.8 with sodium hydroxide. (2) 10% trichloracetic acid. (3) 0.1% 2,4-di-nitro-phenylhydrazine in 2 N HCl. (4) Ethyl acetate. (5) 10% sodium carbonate. (6) 2 N NaOH.

Procedure: Three ml. whole blood (drawn into a tube containing sufficient iodoacetate to give a final concentration of 0.2%) are added slowly with continual shaking to 12 ml. 10% trichloracetic acid in an Erlenmeyer flask. Tissue suspensions from Warburg flasks are added to 4 volumes of trichloracetic acid. After standing for a few minutes the precipitate is filtered or centrifuged off.

Three ml. of the clear filtrate (or supernatant fluid) are added to 1 ml. of the 2,4-di-nitro-phenylhydrazine solution in a conical centrifuge tube. After standing at room temperature for 10 minutes, 4 ml. of ethyl acetate are added and the two layers are mixed (preferably by bubbling a stream of nitrogen through a capillary pipette whose tip rests lightly on the bottom of the tube). After mixing, the layers are allowed to separate and the lower one (water) is carefully drawn off (with the same pipette) and transferred to a second centrifuge tube. The aqueous layer (in the second centrifuge tube) is extracted twice with 2 ml. portions of ethyl acetate and the extracts added to the 4 ml. of ethyl acetate in the original tube. The aqueous layer should now be colorless and may be discarded. The combined ethyl acetate extracts are treated with exactly 2 ml. of 10% sodium carbonate. The layers are mixed (preferably with nitrogen) for several minutes. After the layers have separated the sodium carbonate layer is quantitatively transferred to another tube and the extraction of the ethyl acetate repeated twice using exactly 2 ml. of sodium carbonate each time. The combined sodium carbonate extracts are then extracted once with 1 ml. of ethyl acetate, the latter removed, and the carbonate extract transferred to a colorimeter tube. Four ml. of 2 N NaOH are added and the contents mixed. The color is read in 10 minutes in a photoelectric colorimeter with a filter having the maximum transmission at 520 mμ. Range: 5 to 35 micrograms. Precision: ± 2 micrograms.

A calibration curve is obtained by the use of freshly distilled pyruvic acid (see page 138 and page 208) as a standard. Where large numbers of samples are to be analyzed, a series of tubes fitted into an aeration train arranged so that nitrogen can be bubbled through them all simultaneously is convenient.

If acetoacetic acid is present in the material to be analyzed it may be eliminated by adding 1/10 of its volume of concentrated HCl to the protein-free filtrate and heating on a boiling water bath for 1 hour (Elgart and Nelson, 1941). Concentrated NaOH (equivalent to the HCl) is then added, the solution cooled, and analyzed as described.

Pyruvic acid also may be estimated colorimetrically by the salicylaldehyde method of Straub (1936) or manometrically using carboxylase (see Chapter 14).

METHOD FOR CITRIC ACID

This compound has always been most difficult to estimate. It may, however, be determined colorimetrically with a rather high degree of accuracy. The citric acid is oxidized under controlled conditions with potassium permanganate in the presence of bromine, and is thus converted into pentabromoacetone; this may be measured by the color produced upon addition to sodium sulfide. Pucher, et al. (1934, 1936, 1941) and Purinton and Schuck (1943) have proposed specific quantitative methods based on this principle. All of these require the quantitative extraction of the pentabromoacetone and employ other rather involved procedures. The following method is somewhat simpler and only a single extraction is made. A single extraction was found to remove a constant amount of the total pentabromoacetone in any series of samples of uniform volume. The method is described by Perlman, Lardy and Johnson (1944).

Reagents: (1) Sulfuric acid: equal volumes of 95% sulfuric acid and water. (2) 1 M KBr. (3) Saturated bromine water. (4) 3% H_2O_2. (5) Pentroleum ether (acid washed "Skelly Solve B"). (6) Dioxane-water mixture (equal volumes of dioxane and water). (7) Sodium sulfide solution (4 gms. $Na_2S \cdot 9H_2O$ per 100 ml. solution). (8) 1.5 N potassium permanganate. (9) "Weak" permanganate (0.1 N).

Preparation of samples: If the samples are known to contain reducing materials, aliquots (preferably containing less than 25 mg. citric acid) are placed in 1" x 8" pyrex test tubes, 2 ml. sulfuric acid is added and the total volume is adjusted to about 20 ml. After boiling for a few minutes, the solutions are cooled and 3-5 ml. of bromine water is

added. After 10 minutes any precipitate which may form is removed by centrifugation. The supernatant liquid is decanted off and made to 25 ml. If the samples do not contain appreciable amounts of reducing materials this treatment may be omitted.

Procedure: Aliquots of the sample (containing between 0.2 to 2.0 mg. citric acid in a volume of 3.5 ml. or less), are placed in test tubes (the 18 by 150 mm. size is convenient) graduated at 5 and at 10 ml. Add 0.3 ml. sulfuric acid, 0.2 ml. KBr and 1 ml. of the 1.5 N permanganate and adjust the total volume to about 5 ml. The tubes are allowed to stand for 5 minutes at room temperature and then chilled in an ice bath. The excess permanganate is decolorized with hydrogen peroxide (care must be taken to keep the reaction mixture below 5° C. during this step). Any excess peroxide is removed with "weak" permanganate. The total volume is now adjusted to exactly 10 ml. and 13 ml. of petroleum ether is added. The tubes are stoppered, shaken vigorously and centrifuged (to break any emulsion that may form). Ten ml. portions of the petroleum ether extract are added to colorimeter tubes containing 5 ml. water-dioxane and 5 ml. sodium sulfide solution. The colorimeter tubes are stoppered, shaken vigorously and centrifuged. The color produced should be a light yellow and will be fully developed in 5 minutes. It is stable for several hours. The absorption (in the aqueous bottom layer) is measured at 400-450 mμ., usually the 420 filter is used. A tube containing no citric acid, but which has gone through exactly the same procedure is used as a blank. The content of citric acid is calculated from at least two standards (at different levels) which are run with each set of samples. Range: 200-1800 micrograms citric acid. Precision: ± 10 micrograms.

Precautions: If too large a sample of citric acid has been used, a somewhat red (rather than yellow) color will be developed. In this case a smaller aliquot of the petroleum ether may be added to the dioxane-water-sulfide thus avoiding another complete analysis. When only small quantities of the material are available for analysis, the preliminary acid and bromine treatment may be carried out in a volume of less than 5 ml. in which case the whole sample may be treated with permanganate. The following are critical points in the procedure: (1) An excess of H_2O_2 or permanganate must not be present in the solution before the petroleum ether extraction. Excess H_2O_2 gives low recoveries, excess permanganate gives high recoveries. (2) The solutions must be thoroughly chilled before the excess permanganate is removed or erratic results will occur. (3) Some stabilizing agent must be present during the formation of the colored reaction product of the pentabromoacetone and the sodium sulfide. Both 50% dioxane-water and 50% pyridine-water have proven satisfactory. (4) Sometimes the petroleum ether contains interfering materials. These can be removed by washing with acid. (5) The pentabromacetone should not be allowed to remain in the petroleum ether for more than 15 minutes.

Isocitric acid, cis-aconitic acid, trans-aconitic acid, oxalacetic acid, and gluconic acid do not interfere with this method.

H. A. Lardy

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Chapter XIV

MANOMETRIC ESTIMATION OF METABOLITES AND ENZYME SYSTEMS

INTRODUCTION

The popular usage of manometric equipment for measuring the overall metabolic gas exchange of biological systems has overshadowed to a considerable extent the great usefulness of this equipment for more specific chemical determinations. As has been previously pointed out, any reaction which results in either the production or utilization of a gas can be followed with great accuracy using a manometric apparatus. Further, reactions which give rise to end products having acidic groups can be accurately followed by allowing the reaction to take place in a bicarbonate medium.

In most manometric experiments involving biological transformations the quantity of metabolite involved is usually of the order of 0.5 to 5 mg. The manometric equipment is particularly suitable for semi-micro determinations in this range.

The recent development of micro-colorimetric methods and equipment has tended to replace older manometric methods. In many cases this represents a welcome improvement. However, there still remain a large number of metabolites which are peculiarly suited to manometric estimation. This is particularly true where specific enzyme systems can be utilized. Thus, the important metabolite succinic acid can be accurately determined in small quantities only by means of a manometric method employing a succinoxidase preparation. In certain instances one may have a choice of an equally suitable colorimetric or manometric method. For example, urea can be readily determined colorimetrically (Archibald, 1945) or manometrically with urease at pH 5 (Krebs and Henseleit, 1932). The choice of one or the other method will be determined by the type of equipment available, the experimental set-up, etc. In the author's experience with studies of urea synthesis it was found to be more convenient to use the manometric technique, since the estimation could be carried out directly in the same vessels after removal of the tissue. Obviously one's decision to use one or the other analytical method will be determined to a considerable extent by the experience of the investigator, the type and amount of equipment available, the accuracy requirements of the experiment, etc.

SUCCINIC ACID

Principle: This method was first worked out by Szent-Gyorgyi and Gozsy (1935) and simplified and improved by Krebs (1937). In principle the method depends upon the extraction of succinic acid by means of ethyl ether. The succinic acid is then oxidized by means of a succinoxidase preparation and the O_2 consumption measured. The following reaction takes place (55):

$$(55)\quad COOH\text{-}CH_2\text{-}CH_2\text{-}COOH + \tfrac{1}{2}\,O_2 \longrightarrow COOH\text{-}CH = CH\text{-}COOH + H_2O$$

The specificity of the method is insured by (1) washing the succinoxidase preparation free of coenzymes, and (2) the insolubility in ethyl ether of coenzymes present in the biological system.

Preparation of the sample for extraction: It is desirable to add a deproteinizing agent to the sample before extraction in order to avoid emulsification at the ether-water interface. Sulfuric acid plus sodium tungstate usually has been employed. However, Krebs and Eggleston (1948a) recently have pointed out that in the presence of phosphate ions, excess tungstic acid is converted to phosphotungstic acid which is extractable with ether and which is a powerful inhibitor of succinic dehydrogenase. The formation of inhibitory quantities of phosphotungstate is readily prevented by avoiding an excess of tungstate.

Krebs and Eggleston recommend a preliminary determination of the minimum amount of sodium tungstate required to give a protein free filtrate using an aliquot of the material being studied. Where the succinic content is low it is best to deliver the total sample into the extraction apparatus and then add the deproteinizing reagents and extract directly. To insure an adequate acidity, it is necessary to add an excess of H_2SO_4, usually 1-2 ml. of a 10% solution.

Extraction with ethyl ether: It is essential that the ethyl ether be freed of peroxides before using. This is best accomplished by storing a large quantity of ether over metallic sodium and freshly distilling portions as needed.[1] This involves little trouble if a condenser and a distillation and receiving flask are permanently fixed at the steam bath used for the extraction apparatus. Such a set-up allows for efficient recovery of the ether remaining after the completion of the extraction. The removal of peroxides is essential since the succinoxidase preparation has potent catalase activity, and the liberation of oxygen by this system will interfere in the manometric estimation of oxygen consumption.

A simple and efficient extraction apparatus is that of Kutscher and Steudel as illustrated in Fig. 55. The extractors may be made to contain different volumes of from 15 to 50 ml. The latter size has been found convenient and adaptable for extracting volumes of from 10-35 ml. Since the efficiency of extraction is determined in part by the height of the aqueous column, funnels of different diameters can be employed. Thus with small aqueous volumes, a funnel of wide diameter when filled with ether will heighten the aqueous column outside the funnel very considerably and so enhance the extraction efficiency. The efficiency of extraction is determined by such other factors as the rate of boiling, the temperature, the fineness and speed of the ether bubbles passing through the aqueous layer, etc. It is essential that the efficiency of extraction be determined for each extractor by estimating the recovery of known amounts of succinic acid. As an aid in estimating the rate of extraction, indicators with suitable partition coefficients may be employed (see Krebs, Smyth and Evans, 1940). The author has found that the addition of a few drops of phenol red (0.01% aqueous) to the fluid to be extracted will serve not only as an extraction indicator (about 60 per cent of the succinic acid will have been extracted when the phenol red disappears from the aqueous phase) but in addition serves as a neutralization indicator in the extract. It should be pointed out that at a low pH phenol red has a pink color not unlike that seen in the region of pH 7.

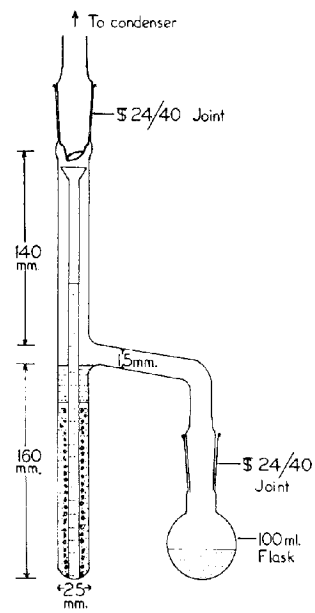


FIG. 55

Kutscher-Steudel extraction apparatus.

When the extraction is completed, 1-2 ml. of 0.1 M phosphate buffer, pH 7.4 is added to the ether solution and the ether distilled off. The last traces of ether are removed by concentrating the aqueous residue on the steam bath to approximately 0.5 to 1 ml. The residue is then transferred to a small graduated cylinder (a graduated 15 ml. centrifuge tube is convenient) by means of a 1 ml. pipette. The flask is rinsed several times with small volumes (0.2-0.5 ml.) of 0.1 M phosphate buffer and the washings added to the contents of the graduated tube. Since the solution in the flask is colored due to the presence of the

[1] The storage flask of ether plus sodium can be conveniently closed off by means of a "Bunsen valve". This is merely a short piece of rubber tubing sealed at one end with a tight fitting glass rod. The rubber tubing is slit with a razor blade, and then fitted over the glass tubing projecting from the cork stopper of the flask. When the pressure inside the flask increases it will permit escape of the gases (hydrogen or ether vapor) through the slit in the tubing.

phenol red, the disappearance of the color in the successive washings can serve as a guide in determining the completion of transfer. The solution in the tube is now adjusted to the proper pH, i.e., 7.4, by the dropwise addition of dilute NaOH, if necessary. The final volume of the extract should be adjusted according to the succinic acid concentration. Since 1 ml. aliquots are usually employed, it is desirable that this amount should contain between 0.2 and 1 mg. of succinic acid. This would represent an uptake of 19 and 95 µl. of O_2 respectively, which is a convenient range.

Preparation of succinoxidase: A simple and suitable succinoxidase preparation can be made as follows: Pigeon breast, pig or sheep heart muscle is freed of fat and connective tissue. It is then coarsely ground in a meat chopper and suspended in 10 volumes of ice-cold distilled water. The suspension is frequently stirred during the first 10 minutes and then allowed to settle in the cold room. The supernatant is decanted and the residue sucked through a double layer of cheesecloth on a Buchner funnel. The muscle pulp is then resuspended in cold distilled water and the procedure is twice repeated. After the third washing, the muscle pulp is dried as completely as possible by suction. For use, a portion of the pulp is suspended in four to five times its weight of 0.1 M phosphate buffer (pH 7.4). This is best accomplished by grinding with a glass mortar and pestle. For storage, the muscle pulp is placed in a tightly covered container and allowed to freeze solid in the freezing compartment of a refrigerator. In this state the preparation remains active and with a low blank O_2 uptake for several weeks (Cohen, 1940a). In this connection it should be pointed out that freshly dissected pigeon breast or freshly killed mammalian heart muscle can be stored for months if frozen solid in covered containers and still yield very active succinoxidase preparations. Where fresh tissue is not readily available at all times this procedure is recommended to insure a continuous supply of enzyme. The phosphate suspension of the muscle pulp develops an appreciable blank O_2 uptake after 24 hours. Since the suspension is rather pasty, it is necessary to pipette it with a wide mouthed pipette. This is best accomplished by breaking off the fine tip from a 3 ml. pipette, flaming it, and then recalibrating to deliver 3 ml.

A dry preparation of succinic dehydrogenase has been described by Weil-Malherbe (1937). Brilliant cresyl blue is used as a carrier.

Manometric estimation of succinic acid: Warburg flasks of about 20 ml. capacity with a center well and a sidearm of 1 ml. capacity are usually employed. To the center well is added 0.2 ml. of 10% KOH plus a square of filter paper. The succinic acid solution, usually 1 ml., is added to the sidearm. The succinoxidase suspension, usually 3 ml. is pipetted into the main compartment. The control vessel is made up in the same way excepting that 1 ml. of 0.1 M phosphate buffer, pH 7.4 with a drop of phenol red, is placed in the sidearm. The bath temperature is usually 40° C. After a 10 minute shaking period with the stopcocks open for equilibration, the manometer fluid is adjusted so as to provide a maximum scale for reading and the stopcocks are then closed. Readings are then taken every 5 minutes until the O_2 uptake is constant in the different manometers. This may require anywhere from 1 to 5 successive readings depending on the temperature of the solutions, the rate of shaking, the thickness of the succinoxidase suspensions, etc. After equilibration is attained, the content of the sidearm is delivered into the main compartment and the manometers swirled once or twice to insure mixing. The manometers are then tipped back and forth once or twice to insure mixing of the solution remaining in the sidearm with the enzyme suspension. A small amount of the suspension is left in the sidearm to insure oxidation of the last traces of the succinic acid. Readings are then taken every 10 minutes until the Δ values (uptake per unit time) of the control and the experimental manometers are equal on two successive readings. The reaction is usually complete in 40 minutes. However, in the presence of high salt concentrations, high concentrations of fumaric, α-ketoglutaric and oxalacetic acids, the reaction rate is slowed up so that periods as long as 90-120 minutes may be required to complete the reaction.

Determination of succinic acid in the presence of malonic acid: It is usually necessary to add malonic acid to aerobic biological systems in which succinic acid synthesis is to be determined. This substance inhibits the oxidation of succinic acid. It is therefore necessary to remove the malonic acid before the succinic acid determination can be carried out. This is most simply done by oxidation with acid permanganate using a procedure similar to that employed for α-ketoglutaric acid determination (see page 169).

Malonic acid is readily oxidized by acid permanganate while succinic acid is not. If α-ketoglutaric acid is present in this system, which will be likely, it will be converted to succinic acid by the permanganate treatment and therefore will be included in the succinic acid determination. Should it be desired to determine succinic acid only, it is possible to remove the succinic and malonic acids from the α-ketoglutaric acid by the addition of $NaHSO_3$ in slight excess, and enough H_3PO_4 to make the solution 0.04 N. Since the sulfite addition product of α-ketoglutaric acid is relatively insoluble in ether, the succinic and malonic acids can be extracted with ether (Weil-Malherbe, 1937). After removal of the ether the malonic acid can be destroyed by oxidation with acid permanganate, leaving succinic acid (Krebs and Eggleston, 1940).

Analytical range: The smallest quantity of succinic acid which can be determined by the manometric method is limited chiefly by the accuracy of the manometric equipment. Since 0.05 mg. of succinic acid is equivalent to 4.75 µl. of O_2 uptake, this amount can be considered the lower limit of the method.

Calculation:

µl. O_2 uptake is converted to mg. of succinic acid as follows

$$\frac{\mu l.\ O_2}{112} \times 1.18 = \text{mg. succinic acid}$$

It should be noted that 1 µl. O_2 uptake is equivalent to 2 µl. succinic acid.

α-KETOGLUTARIC ACID

Principle: This method depends on the conversion of α-ketoglutaric acid, either as such or in the form of its dinitrophenylhydrazone, to succinic acid by oxidation with acid permanganate (Krebs, 1938) (Equation 56). The succinic acid formed is then determined by means of a succinoxidase preparation as described in the previous section.

(56) $COOH\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}COOH \longrightarrow COOH\text{-}CH_2\text{-}CH_2\text{-}COOH + CO_2$

Reagents:
1) 50% H_2SO_4.
2) 10% Na_2WO_4.
3) 0.8 N H_2SO_4.
4) 3% $KMnO_4$.
5) 2:4 dinitrophenylhydrazine, 1% dissolved in 10% H_2SO_4.
6) Reagents for succinic acid determination.

Procedure: If appreciable quantities of interfering substances are present (see under specificity) α-ketoglutaric acid is converted to its dinitrophenylhydrazone (see below) and extracted with ether as outlined by Krebs (1938). This is unnecessary with many biological systems since the concentration of metabolites which might interfere is not significantly great. In most experiments, therefore, one may proceed as follows. An aliquot of the deproteinized solution is delivered directly into the Kutscher-Steudel extractor, followed by 1 ml. of 50% H_2SO_4 plus 2 ml. of 3% $KMnO_4$. The solution is allowed to stand at room temperature for 30 minutes. If during this time the permanganate is decolorized, more is added. The solution is then directly extracted with ether. The ether extract usually contains considerable quantities of MnO_2. This, however, does not interfere with the succinic acid determination but, if desirable, can be readily removed by centrifugation after the ether is removed and the solution made up to volume. The procedure otherwise is the same as that outlined for succinic acid.

A succinic acid blank is determined by ether extraction of the protein free filtrate without permanganate treatment. When high concentrations of substrates, which react with permanganate to yield succinic acid are employed, it is necessary to run either a "zero

time" blank with this substrate present, or to separate the α-ketoglutaric acid as its dinitrophenylhydrazone.

For separation of α-ketoglutaric acid as its dinitrophenylhydrazone, the following procedure is employed. To an aliquot of the protein-free filtrate is added 1-2 ml. of 1% dinitrophenylhydrazine solution in 10% H_2SO_4. The solution is allowed to stand for 30 minutes and then extracted twice with 1/5 volume of ether in a separatory funnel. The ethereal solutions are combined and the ether removed by evaporation on the steam bath. The residue is dissolved in 2-5 ml. of 2 N NaOH and transferred quantitatively to a graduated cylinder of 25-50 ml. capacity. The solution (usually about 20 ml.) is acidified with 50% H_2SO_4 to bring the acidity to about 1 N. 3 ml. of 3% $KMnO_4$ are then added and the solution allowed to stand at room temperature for about 30 minutes. If decolorization of the permanganate occurs during this interval, more permanganate solution (or solid if the volume is to be kept within certain limits) is added. The final volume is then determined, the solution filtered and the succinic acid extracted with ether in the usual manner from an aliquot of the filtrate.

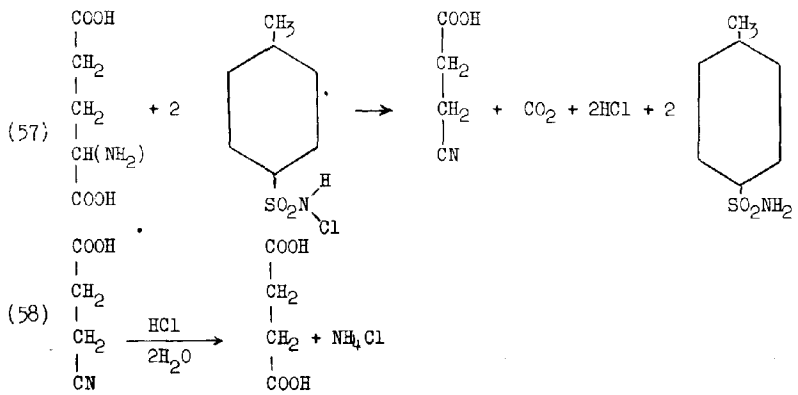
Specificity: Substances which yield appreciable amounts of succinic acid when oxidized with acid permanganate are α-hydroxyglutaric acid, arginine, butyric acid and glutamic acid. Krebs and Eggleston (1948a) recently have pointed out that while glutamic acid in pure solution does not yield significant amounts of succinic acid, in the presence of deproteinized tissue homogenate up to 70 per cent of theoretical yields may be realized. Since glutamic acid is present in considerable concentration in many tissues, it is necessary to separate the α-ketoglutaric acid as the dinitrophenylhydrazone as previously described or to extract the α-ketoglutaric acid with ethyl ether and then proceed with the $KMnO_4$ oxidation. If significant quantities of the other interfering substances are present in the preparations to be analyzed, the separation of α-ketoglutaric acid either as the dinitrophenylhydrazone or by means of ethyl ether, is indicated.

Calculation: The ul. O_2 uptake is converted to mg. of α-ketoglutaric acid as follows:

$$\frac{\mu l.\ O_2}{112} \times 1.46 = \text{mg. of } \alpha\text{-ketoglutaric acid}$$

GLUTAMIC ACID

Principle: This method depends upon the conversion of glutamic acid by means of an excess of chloramine-T to β-cyanopropionic acid, and the hydrolysis of the latter to succinic acid according to equations (57) and (58) (Cohen, 1939). The succinic acid is then determined by means of a succinoxidase preparation as previously described.



Reagents:

1) Citrate buffer, pH 4.7. (17.65 grams $Na_3C_6H_5O_7 \cdot 2H_2O$ and 8.40 grams of $C_6H_8O_7 \cdot H_2O$ are dissolved in H_2O and diluted to 50 ml.)
2) 0.1 M phosphate buffer, pH 7.4. (17.8 grams $Na_2HPO_4 \cdot 2H_2O$ are dissolved in about 500 ml. of H_2O and 20 ml. of 1 N HCl added. The solution is then diluted to 1 L.)
3) 10% chloramine-T (N-chloro-p-toluenesulfonamide) must be freshly prepared before use.
4) Conc. HCl.
5) 5% NH_4Cl solution.
6) Sat. NaOH solution (50%).
7) Reagents for succinic acid determination.

Procedure:

1) Deproteinization
When tissue slices are employed it is not necessary to deproteinize since the small amount of protein present does not interfere. Tissue minces, homogenates, etc., are deproteinized with H_2SO_4--Na_2WO_4 solutions. An aliquot of the filtrate is used for the determination.

2) Oxidation by chloramine-T
The solutions to be analyzed are brought to pH 4.7 by the addition of 1-1.5 ml. of citrate buffer. 2 ml. of freshly prepared 10% chloramine-T are added and the solutions well mixed by shaking. They are then placed in a rack and shaken at 40° for 10 minutes. The reaction is conveniently carried out in small Erlenmeyer flasks, or, where tissue slices are employed, the reaction may be carried out in the manometric flasks after removal of the slices and the alkali in the center well. After 10 minutes shaking the containers are removed and placed in an ice bath for 15-20 minutes to precipitate most of the p-toluene-sulfonamide formed as a reaction product, and most of the unused chloramine-T. The solutions are filtered while cold, the precipitate washed with several small volumes of cold water, and the combined filtrates and washings collected in large test tubes (25 x 200 mm.).

3) Hydrolysis of β-cyanopropionic acid
Conc. HCl is added to the filtrate to make a final concentration of not less than 12.5%. The tubes are covered and placed in a boiling water bath for 15 minutes after which time they are removed and allowed to cool. Conc. NaOH is added dropwise until the solution becomes hot. At this point 0.5 ml. of 5% NH_4Cl solution is added and the contents well mixed. The NH_4Cl decomposes traces of chloramine-T which if present will decolorize the indicator. The solution is cooled and a few drops of phenol red are added. The solution is then made alkaline to a purple color. A large excess of alkali should be avoided as the p-toluene-sulfonamide forms a salt in strongly alkaline solution. The solution is then transferred to a Kutscher-Steudel extractor and extracted with ether. The alkaline solution is extracted with freshly distilled ether for a time sufficient to remove the remaining traces of p-toluene-sulfonamide (usually 1-2 hours). After this time, the extraction flasks are removed and replaced by clean ones. The contents of the extractors are then acidified with 2-3 ml. of 10% H_2SO_4. The phenol red will change to a light yellow-pink color. Additional ether is then added and the extraction resumed for two hours or more. (See discussion under succinic acid determination.)

Preparation of solution for succinic acid determination: Essentially the same procedure is followed as that outlined under succinic acid determination.

Calculation: The µl. O_2 uptake is converted to mg. of glutamic acid as follows:

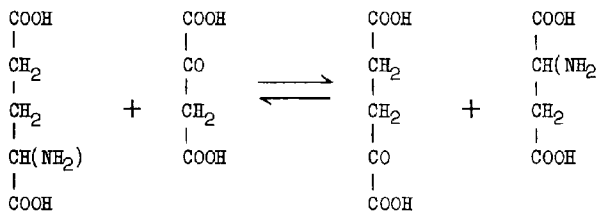
$$\frac{\mu l.\ O_2}{112} \times 1.47 = \text{mg. glutamic acid}$$

Specificity of the method: Aside from succinic, only glutamine and glutathione are known to interfere with the determination of glutamic acid. Succinic acid is readily taken care of by extraction of the solution without chloramine-T treatment. This will represent a succinic acid blank. Since glutamine will be encountered in appreciable quantities only under rather special conditions (Krebs, 1935a) this substance will present few difficulties. Glutathione, on the other hand, is present in fairly high concentration in most tissues. In experiments where glutamic acid formation or disappearance is being measured, a "zero time" blank will take care of the glutathione content of the tissue. However, if the absolute concentration of glutamic acid is desired, it is necessary to separate the β-cyanopropionic acid from the glutathione homologue by ether extraction. For details of this, consult the original paper (Cohen, 1939).

ASPARTIC ACID FORMATION AND DISAPPEARANCE IN TRANSAMINATION SYSTEMS

As can be seen from Reaction (57), glutamic acid yields 1 mole of CO_2 when it reacts with an excess of chloramine-T at an acid pH. This is true of most of the other amino acids with the exception of glycine and aspartic acid which yield 2 moles of CO_2 (Cohen, 1940b). Since transamination is concerned chiefly with the following reaction (59), it is possible to

(59)



determine aspartic acid formation and disappearance in transaminating systems with the above substrate combinations. For purpose of rapid assay of any tissue or cells for transaminase activity, or for following activity in purification procedures, the method is both accurate and rapid. For analytical details the reader is referred to the paper by Cohen, (1940b). This method has been used for study of transamination in animal tissues (Cohen and Hekhuis, 1941), in plant tissues (Albaum and Cohen, 1943) and in bacteria (Lichstein and Cohen, 1945 and Cohen and Lichstein, 1945).

FUMARIC ACID

Principle: (Krebs, Smyth and Evans, 1940). Fumaric acid is reduced to succinic acid in the presence of zinc and phosphoric acid (60),

(60) $COOH\text{-}CH = CH\text{-}COOH \xrightarrow{H_2} COOH\text{-}CH_2\text{-}CH_2\text{-}COOH$

The succinic acid formed is extracted with ether and determined manometrically by means of a succinoxidase preparation.

Reagents:

1) Meta-phosphoric acid, 5% solution (made up without heating; can be stored in ice box for 1-2 weeks).
2) Zinc filings, 20-30 mesh.
3) $CuSO_4 \cdot 5H_2O$, 20% solution.
4) 10 M phosphoric acid solution (100 ml. phosphoric acid, sp. gr. 1.75, plus H_2O to 158 ml.).
5) Reagents for succinic acid determination.

Procedure: Deproteinization is carried out by the addition of 1/5 volume of 5% metaphosphoric acid. An aliquot of the filtrate is transferred to a Kutscher-Steudel extractor, or a measuring cylinder, and 0.5 grams of zinc filings, 2.3 ml. of phosphoric acid and 0.25 ml. of $CuSO_4$ solution are added per 10 ml. aliquot. (Excessive frothing can be controlled with a drop of capryl alcohol.) After 60 minutes, when the greater part of the zinc has been decomposed, the formed succinic acid is extracted directly with ether. The succinic acid so extracted is then estimated as outlined previously.

Calculations: The µl. of O_2 uptake is converted into mg. of fumaric acid as follows:

$$\frac{\mu l.\ O_2}{112} \times 1.16 = \text{mg. fumaric acid}$$

Specificity: Malic, tartaric, oxalacetic, aspartic, glutamic, citric and aconitic acids do not form succinic acid under the conditions of this method. Maleic acid behaves like fumaric acid, but since it is not present in biological material it need not be considered. If the solution contains succinic acid, it must be determined separately in an aliquot before the treatment with zinc and acid and deducted from the succinate found in the fumaric acid determination.

The use of the equilibrium constant for the reaction

$$COOHCH{=}CHCOOH \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} COOHCH_2CHOHCOOH$$

as previously suggested by Krebs, Smyth and Evans (1940) has proven to be unreliable (Krebs and Eggleston, 1945) when applied in different systems. Thus the use of this method for the determination of malic acid is not recommended.

PYRUVIC ACID

Principle: The manometric estimation of pyruvic acid is based on the production of CO_2 by enzymatic decarboxylation (Warburg, et al, 1930; Westerkamp, 1933) at an acid pH (Reaction 61).

$$(61)\quad CH_3COCOOH \xrightarrow{\text{Carboxylase}} CH_3CHO + CO_2$$

The carboxylase method is particularly suitable for the rapid determination of pyruvate in certain biological systems.

Reagents:

1) Yeast extract

Freshly pressed brewers yeast is spread on filter paper and dried at room temperature with the aid of a fan. If quickly dried and stored in the ice box, the dried yeast will yield active carboxylase preparations for as long as 3 years. For preparation of the extract, 10 grams of dried yeast are suspended in 30 ml. of distilled water by grinding in a mortar. The suspension is then poured into a tall cylinder (100 ml. graduate is suitable) and allowed to stand in a water bath at 30° C. for 1-2 hours, or until the endogenous fermentation has ceased. The suspension is then centrifuged at fairly high speeds for 15-20 minutes. The milky supernatant liquid is removed by decantation. It is treated with 1/20 volume of acetate buffer and freed of dissolved CO_2 by shaking the container while attached to a water vacuum pump. The carboxylase activity of such a preparation decreases rapidly on storage even at 0° C. Stabilization on storage at 0° C. for almost 1 week may be accomplished by adding 1/5 volume of 90% glycerol. (Schoenebeck and Neuberg, 1935). In the author's experience it has been found more expedient to make up fresh preparations as needed.

2) 3 M Acetate buffer, pH approximately 5.
2 parts of $NaC_2H_3O_2 \cdot H_2O$ plus 1 part glacial acetic acid.
1/10 volume of M/2 KH_2PO_4 is added to the buffer.

Procedure: If tissue breis, minces, homogenates, or bacterial suspensions are being used in studying pyruvic acid metabolism, the system is acidified with 1 ml. of acetic acid--acetate buffer, transferred to a graduated centrifuge tube and made up to a convenient volume with washings. Where pyruvic acid disappearance is being studied and a high concentration of pyruvic acid is present, it is necessary that the dilution be great enough so that the CO_2 production from the aliquot will not exceed the capacity of the manometer. Thus, if one were to add 1344 µl. of pyruvic acid (0.3 ml. of 0.2 M solution) to a system and then measure pyruvic acid disappearance, an aliquot of approximately 1/5 the total volume should be taken. This would represent, assuming no disappearance, a production of 269 µl. CO_2. If the expected disappearance is great, the final volume should be kept down accordingly so as to permit the use of a constant volume aliquot, usually 2 ml. In experiments with tissue slices it is only necessary to remove the slices and then add the acetic acid--acetate buffer and make up to volume. With very heavy tissue and bacterial suspensions it is desirable to centrifuge after adding buffer and making up to volume. An aliquot (usually 2 ml.) of the supernatant is then employed.

The manometric flasks are usually set up to contain a 2 ml. aliquot of the acidified incubation system in the main compartment. The sidearm contains 0.5 ml. of the yeast extract. Bath temperature is usually set at 25° C. The control vessel contains 2 ml. of water treated with 1/10 volume of acetic acid--acetate buffer in the water compartment in place of the incubation system.

It is necessary to equilibrate until all CO_2 production ceases, or is constant in both the control and experimental vessels. Readings are taken every 5 minutes after the taps are closed. The solutions are then mixed, and readings again taken every 5 minutes. The reaction is usually complete in 10-15 minutes. As a matter of fact, significant CO_2 production beyond this time usually means that the carboxylase system is weak, or that other substrates, such as ketoglutaric or oxalacetic acids are reacting.

Calculations: µl. of CO_2 produced is converted into mg. of pyruvic acid as follows:

$$\frac{\mu l.\ CO_2}{224} \times 0.88 = \text{mg. of pyruvic acid.}$$

Specificity: Most of the α-ketonic acids will yield CO_2 when added to yeast extract. However, the rates of reaction vary considerably, pyruvic acid reacting at the fastest rate. A comparison of the relative rates for different α-keto acids can be seen from the following Table (XXIX):

TABLE XXIX

Decarboxylation of α-keto acids

Keto acid	Per cent theoretical CO_2 production in 15 minutes
Pyruvic	100
α-ketobutyric	90
α-ketoglutaric	8
oxalacetic	20
acetopyruvic	60

It is apparent from the preceding table that the determination of pyruvic acid in systems containing relatively large amounts of other α-keto acids will present some difficulty. Since α-ketobutyric acid will not normally be present in measurable amounts, this

compound will not be a source of error. However, if one attempts to measure pyruvate in a system where relatively large quantities of oxalacetic acid are present, it may be difficult to obtain accurate pyruvate values by the carboxylase method.

Since the yield of CO_2 from oxalacetic acid is erratic, it may be converted to pyruvic acid by previous heating of the sample, adjusted to 0.4 N HCl, for 15 minutes at 100° (Krebs and Eggleston, 1948b). Before analysis the pH is adjusted back to 5 with 3 M sodium acetate solution. This technique will be found to be particularly valuable in experiments in which the chief reaction product is pyruvic acid (for example the production of pyruvic acid from lactic acid by means of lactic dehydrogenase preparations). As examples of determinations in the presence of considerable concentrations of interfering substances the experiments of Krebs and Eggleston (1945, 1948b) on keto-acid metabolism in different tissues, and those of Cohen (1940 a, b) on transamination may be cited.

OXALACETIC AND ACETOACETIC ACIDS

Principle: This method is based on the fact that β-keto acids are catalytically decomposed by primary amines in an acid medium to yield CO_2 (Reaction 62).

$$(62)\quad RCOCH_2COOH \longrightarrow RCOCH_3 + CO_2$$

Ostern (1933) first applied this principle to the determination of oxalacetic acid using aniline as a catalyst. The reaction was carried out in acetic acid -- acetate buffer, pH 5, and at 5° C. Quastel and Wheatly (1933) introduced the use of aniline hydrochloride for the determination of aceto-acetic acid. Edson (1935) employed aniline citrate for the same reaction, to better advantage. The use of aniline citrate for oxalacetic acid determination was reported by Greville (1939). The advantage of aniline citrate over that of other aniline salts is the greater solubility of this compound which insures a high concentration of the catalyst.

While aniline decarboxylates both acetoacetic and oxalacetic acids, Al^{+++} salts decarboxylate oxalacetic acid only (Krebs, 1942a; Krebs and Eggleston, 1945). However, in the presence of pyruvate, CO_2 may be liberated from acetoacetic acid (or a condensation product) by aluminum ions (Krebs and Eggleston, 1948b).

Reagents:

0.75 M phthalate buffer - 15.3 gm. potassium hydrogen phthalate and 1.8 gm. NaOH in 100 ml.

Aluminum sulfate - 33.3 gm. $Al_2(SO_4)_3 \cdot 1\ H_2O$ per 100 gm. H_2O.

Aniline citrate - 4.5 ml. freshly distilled aniline plus 5.5 ml. of 50 percent citric acid.

2 N HCl

Procedure: At the end of the incubation period, 0.25 volumes of 2 N HCl are added to the flasks which are then placed in an ice bath. Slices are removed at this time in the usual manner. In the case of tissue minces or homogenates, the cold, acidified solutions are centrifuged and suitable aliquots are taken for analysis. The rate of spontaneous decomposition of the β-keto acids is lowered by the use of mineral acids. Thus Krebs and Eggleston (1945) recommend the addition of 2 N HCl to the solution to be tested to bring the final concentration to about 0.2 N.

Determination of Acetoacetic Plus Oxalacetic Acids: The main compartment of the vessels is filled with 3 ml. of the unknown solution, 0.3 ml. of 2 N HCl and 0.5 ml. of 50 percent citric acid, the sidearm with 1 ml. of aniline citrate. The determination is best carried out at low temperatures; however, 20-25° C. is satisfactory. An initial equilibration period of 5 minutes with the taps open is necessary to insure removal of dissolved CO_2. After the taps are closed, readings are taken every 5 minutes until constancy is reached. The flask contents are then mixed. Readings are then taken as usual until no more CO_2 is produced, or until the pressure changes in the control and experi-

mental flasks are constant. Immediately on mixing a negative pressure will usually result due to the difference in density of the aniline citrate solution and the solution in the main compartment. This is corrected for by the use of a control vessel, which contains water in place of β-keto acid.

Determination of Oxalacetic Acid: Warburg flasks with double sidearms each having a capacity of 1 ml. are used. The main compartment contains 2 ml. of the solution to be tested (previously acidified to approximately 0.2 N with 2 N HCl). One sidearm contains 1 ml. of 0.75 M phthalate buffer and the other 1 ml. of 33.3 per cent aluminum sulfate solution. A bath temperature of 20-25° C. may be used. After equilibration, the phthalate buffer is introduced from the sidearm followed by the aluminum sulphate solution. A control experiment using water in place of the unknown solution is necessary in order to correct for the pressure changes caused by mixing the different solutions. The CO_2 evolution is usually complete within 60 minutes. Recovery experiments give yields approximately 5 percent below theory due to spontaneous decomposition of oxalacetic acid during equilibration. Acetoacetic acid is determined by difference between the aniline citrate and the aluminum sulfate values.

Calculations: Microliters of CO_2 produced is converted to mg. of oxalacetic and acetoacetic acids as follows:

$$\frac{\mu l\ CO_2}{224} \times 1.32 = \text{mg. of oxalacetic acid}$$

$$\frac{\mu l\ CO_2}{224} \times 1.02 = \text{mg. of acetoacetic acid}$$

Specificity: Aniline salts will catalyze the decomposition of all β-keto acids. Aluminum ions (and other multivalent cations) on the other hand catalyze the decomposition chiefly of β-ketodicarboxylic acids and have no effect on α- or β-keto monocarboxylic or α-ketodicarboxylic acids.

XANTHINE AND HYPOXANTHINE

Principle: (Krebs and Orstrom, 1939). Xanthine and hypoxanthine are oxidized to uric acid by means of a purified xanthine oxidase preparation. O_2 uptake and uric acid formation are measured. From the ratio of O_2 uptake to uric acid formation the amounts of xanthine and hypoxanthine can be calculated. The former compound requires 2 atoms of oxygen, the latter, 1 atom for oxidation to uric acid.

Reagents:

1) Xanthine oxidase: This preparation must be highly active. A suitable preparation is made from fresh milk as follows (Dixon and Kodama, 1926): a liter of milk is clotted with renin and the clot broken up with a glass rod. The whey is filtered off through muslin. Solid ammonium sulfate is added to the amount of 215 grams for each 880 ml. of whey. After standing for 30-60 minutes the underlying clear solution is sucked off as completely as possible. The supernatant globulin layer is now centrifuged and the solid floating cake transferred to a filter paper and dried in vacuo. The dried material is freed of fat by ether extraction and the residue dried again. The enzyme keeps for several weeks if stored in vacuo. The yield is usually of the order of 3-5 grams per liter of milk. For use, the enzyme is made up as a 10% solution in 0.1 M phosphate buffer, pH 7.4. The activity of the preparation should be tested with a standard hypoxanthine solution. 15 mg. of the enzyme (0.15 ml. of 10% solution) should be capable of oxidizing 0.5 mg. of hypoxanthine in less than 1 hour (40°, pH 7.4).

2) Aqueous Pigeon liver extract: 1 gram of pigeon liver is thoroughly ground with sand and 10 parts of water and centrifuged. The supernatant liquid containing an active catalase, is used. The solution if preserved with octyl alcohol and stored in the refrigerator will remain active for several weeks. Mammalian liver is not suitable since it may contain uricase.

3) Phosphate buffer, 0.1 M, pH 7.4.

Procedure: The sample to be analyzed is adjusted to pH 7.4. (This need be approximate only, since the xanthine oxidase activity does not vary significantly between pH 6.8 and 7.8.) The main compartment of the manometric flask contains 0.5 ml. of phosphate buffer, 0.5 ml. of liver extract, 3.5 ml. of the solution to be tested, and 0.15 ml. of xanthine oxidase solution. The center well contains 0.2 ml. of 10% KOH. As a blank, a flask is set up which contains all the constituents except that 3.5 ml. of water is used in place of the solution being tested. The bath temperature is 40°. The O_2 uptake should be complete in less than 60 minutes and should agree with the theoretical value within 2%.

Uric acid may be determined by any one of several methods. Suitable procedures include: (a) conversion of uric acid to urea and determination of the latter manometrically (Edson and Krebs, 1936), and (b) the Folin colorimetric methods using uricase to insure specificity (Blauch and Koch, 1939).

Calculations: On the basis of hypoxanthine requiring 1 mole and xanthine 0.5 mole O_2 for conversion to uric acid, the following equations may be set up:

$$X_{\text{hypoxanthine}} = 2\,X_{O_2} - X_{\text{uric acid}}$$

$$X_{\text{xanthine}} = 2\,(X_{\text{uric acid}} - X_{O_2})$$

(all subscripts refer to moles)

Specificity: Of the many substances which may absorb O_2 in the presence of xanthine oxidase, only hypoxanthine, xanthine, adenine and a few aldehydes are known to occur in biological material. The presence of aldehydes is indicated if an absorption of O_2 but no formation of uric acid occurs. It is unlikely that, in animal tissues at least, aldehydes will be present in quantities sufficient to interfere. Adenine, if present, will be determined as hypoxanthine. However, its presence will be suggested by its slow rate of oxidation. Adenine is oxidized at 1/20 the rate of hypoxanthine.

Since tissue extracts may contain appreciable quantities of nucleotides, nucleosides, or guanine, all of which liberate hypoxanthine or xanthine, the bound purine bodies may represent a potential source of error. In the application of the method by Krebs and Orstrom to pigeon liver preparations, no difficulties on this basis were encountered.

In addition to substances which may react directly with xanthine oxidase preparations, a number of compounds may interfere with the quantitative determination of O_2 uptake due to a coupled oxidation. Alcohols, hemoglobin, methemoglobin, nitrites, etc. react in such a system due to the fact that they are readily oxidized by the hydrogen peroxide formed as an end product of the xanthine and hypoxanthine oxidation. Of these substances hemoglobin and its derivatives are the only ones likely to be present in animal tissues. This coupled oxidation is inhibited within certain limits by the use of liver extract which because of its high catalase activity rapidly decomposes the hydrogen peroxide as it is formed.

The lower limit of the analytical range of this method is 0.1 mg.

UREA

Principle: (Krebs and Henseleit, 1932.) Urea is converted by urease to ammonium carbonate at pH 5. The ammonium carbonate in turn reacts to yield CO_2 according to the following equation (63).

(63) $\begin{matrix}NH_2\\|\\CO\\|\\NH_2\end{matrix}$ $\xrightarrow{\text{urease}}$ $(NH_4)_2CO_3$ $\xrightarrow{2H^+}$ $2(NH_4)^+ + H_2O + CO_2$

The CO_2 formed is measured manometrically.

Reagents:

1) Urease: While urease itself is highly specific in its action, the ordinary sources of urease may contain enzyme systems which will yield CO_2 under similar conditions. In particular, carboxylase may yield CO_2 from pyruvic acid under the above conditions. Thus it has been shown that commercial jack bean meal contains a potent carboxylase system (Cohen, 1946). Soy bean meal extracts, on the other hand, are practically devoid of this enzyme. The carboxylase activity of the jack bean meal extract is readily lost on dialysis against dihydrogen sodium phosphate solution without influencing the urease activity. In practice, however, it has been found far more convenient to purchase a purified urease (Arlco urease, made by Arlington Chemical Co., Arlington, Mass.) which is free of carboxylase. Since this material is stable for a long time and requires no preparation other than solution before use, it is more economical from the standpoint of both time and money. It is best prepared in a concentration of 10 mgs. per ml. of solution, the latter being 9.5 parts distilled water plus 0.5 parts acetic acid--sodium acetate buffer, pH 5.

2) Acetic Acid-Sodium Acetate Buffer: 27.2 g. sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) plus 6 g. glacial acetic acid are made up to 100 ml. with distilled water. This represents a 3 N acetic-ion concentration and has a pH of 5.

Procedure: If tissue slices are used, these are removed from the flasks and the remaining solution acidified with 0.3 ml. of acetic acid-sodium acetate buffer; 0.5 ml. of urease solution is added to the sidearm.

In the case of tissue minces or homogenates, acetic acid-sodium acetate buffer is added directly to the flask and the total volume noted. The preparation is then centrifuged and a suitable aliquot is placed in the main compartment of a clean flask with 0.5 ml. urease solution in the sidearm. The control vessel contains distilled water plus acetic acid-sodium acetate buffer in place of the experimental solution. After a suitable equilibration period, the solutions are mixed and the manometric changes observed. The reaction is usually run at 38° with air as a gas phase. The reaction should be completed in 20-30 minutes.

Calculation: The µl. CO_2 produced is converted to mg. urea as follows:

$$\frac{\mu l.\ CO_2}{224} \times 0.60 = \text{mg. urea.}$$

Specificity: As previously pointed out, in the presence of pyruvate and related substances, high "urea" values may be obtained because of carboxylase activity in the crude urease preparations. By the use of purified urease preparations this difficulty is eliminated. Krebs (1942b) has recently called attention to another source of CO_2 unrelated to urea, i.e., aceto-acetic acid. This substance is found in considerable amounts when liver slices are incubated in the presence of ammonium salts (Edson, 1935). Since aceto-acetic acid slowly decomposes to yield CO_2, it may interfere with the urea determination. In order to destroy the acetoacetic acid, Krebs recommends the addition of 0.1 ml. of aniline plus 1 ml. of acetic acid-sodium acetate buffer to the unknown solution. The aniline catalyzes the decomposition of the acetoacetic acid so that it is completely broken down within the usual equilibration period of 20 minutes. (See method for acetoacetic acid determination.)

GLUTATHIONE

Principle: This method (Woodward, 1935) depends on the fact that the glyoxalase system requires reduced glutathione as a coenzyme. The rate of conversion of methylglyoxal to lactic acid is dependent on the concentration of glutathione within certain limits. The reaction is carried out in a bicarbonate - CO_2 system. The CO_2 formed as a result of the conversion of methyl glyoxal to lactic acid is measured manometrically (Reaction 64).

(64) $CH_3COCHO \xrightarrow[GSH]{H_2O} CH_3CHOHCOOH$

Reagents:

1) Acetone dried yeast: This is prepared according to Albert, et al. (1902). 500 grams of starch-free bakers' yeast is coarsely pulverized and passed through a sieve (10 mesh) into 3 liters of acetone in a flat dish. After stirring, the yeast is allowed to remain in the acetone for 10 minutes. The acetone is then decanted and the residue sucked dry on a hard filter paper. The resulting cake is again broken up and suspended in one liter of acetone for 2 minutes. After decanting the supernatant the yeast residue is sucked dry. The resulting cake is then broken up and covered with 250 ml. of ethyl ether and mixed for 3 minutes after which the suspension is filtered by suction. The yeast cake is finely pulverized and spread out in a thin layer on filter paper for 1 hour. The resulting powder is then further dried by placing in a desiccator for 24 hours at 45°. Before use, the yeast must be freed of glutathione. This is most readily done by suspending 1 gram of dried yeast in 50 ml. of distilled water and centrifuging. The supernatant is decanted and the centrifugate suspended again with 50 ml. of distilled water. After centrifuging again and decanting, the yeast is made up as a 15-20 percent suspension and stored in the ice box.

2) Methyl glyoxal: (Methyl glyoxal, pyruvic aldehyde, is obtainable from Commercial Solvents Corporation as a 30 percent aqueous solution.) A solution of methyl glyoxal is readily prepared by distilling dihydroxyacetone from H_2SO_4 according to Neuberg, et al. (1917). 0.5 grams of dihydroxyacetone is introduced into a 100 ml. distilling flask. A mixture of 5 grams H_2O plus 1 gram of H_2SO_4 is placed in a small separatory funnel which is tightly fixed in the flask neck with a rubber stopper. The distilling flask is connected with a small efficient water-jacketed condenser. The H_2SO_4 solution is added dropwise while the flask is being heated. After 5 ml. of distillate has come over (the distillate is best collected in a glass stoppered graduated cylinder) 5 ml. of distilled water should be added dropwise through the funnel. This procedure is repeated until a drop of the distillate no longer gives a precipitate when added to a dilute acetic acid solution of m-nitrobenzhydrazide. To determine the concentration of methylglyoxal, the method of Friedemann (1927) is both simple and sufficiently accurate. An aliquot is titrated with 0.1 N NaOH to a pink color with phenolphthalein. Neutral 5% H_2O_2 solution is then added followed by a known amount of 0.1 N NaOH. (The amount will be determined by the methylglyoxal concentration. As a guide, the solution should remain pink to phenolphthalein while standing for 10 minutes at room temperature.) The flask is stoppered and allowed to stand at room temperature for 10 minutes. The solution is then titrated with 0.1 N HCl until colorless.

3) 2% sulfosalicylic acid.

4) 95% N_2 - 5% CO_2 gas mixture.

5) 0.2 M Sodium bicarbonate.

Procedure: A standard curve is prepared by adding known amounts of reduced glutathione to the yeast-methyl glyoxal system. The levels of gluthathione suggested are 0.025, 0.05, 0.1 and 0.15 mg. A blank with no glutathione is run and subtracted from these values. The flasks are set up to contain the following in the main compartment: 0.15 ml. of a 15-20 percent yeast suspension; (The amount of yeast to be used is determined by its glyoxalase activity. This is estimated by measuring the amount of CO_2 found in 20 minutes

in the presence of 0.1 mg. of glutathione and 10 mgs. of methyl glyoxal. An amount of yeast should be taken which will yield 200-250 µl. of CO_2 under these conditions); 10 mgs. of methyl glyoxal (usually about 0.5 ml.); 0.4 ml. of 0.2 M sodium bicarbonate; H_2O to make 2 ml. The sidearm holds the glutathione containing solution. If deproteinization is required, this is accomplished with 2% sulfosalicylic acid. The acid filtrate is neutralized before use with 0.2 M sodium bicarbonate to methyl orange. (This is best done with an aliquot.)

After gassing with 95% N_2-5% CO_2 mixture the flasks are placed in a bath (25°) and the manometer fluid levels adjusted so that the level of the left hand column is between 0 and 5 mm. (This is accomplished by sucking gas out of the closed system after gassing and readjusting the level of the right column to 15. By this procedure the capacity of the manometer is almost doubled.) After an initial equilibration period with shaking (during which time the gas production in the control and experimental flasks should be equal) the contents of the side bulbs are tipped in. Readings are taken every 5 minutes without stopping the shaking apparatus. The first 5 minute reading is discarded since it will usually be too high due to mixing effects. Readings are taken for 20 minutes and the first 5 minute value calculated by extrapolation.

Calculation: The glutathione concentration is determined by reading the 20-minute CO_2 value from the standard curve.

Accuracy and Specificity: The accuracy of the method is determined chiefly by the pipetting accuracy of the different reagents. According to Woodward (1935) the limit of error does not exceed 6 percent.

This method is highly specific since no naturally occurring sulfhydryl compound other than glutathione will react in this system. Further, ascorbic acid is not active and thus the procedure can be used in the presence of this reducing substance. While the method does not lend itself to a routine use, it is most valuable where a quantitative estimation of glutathione in biological material is required.

D-AMINO ACIDS

The determination of D-amino acids ("non-natural") is of some interest since they have been shown to occur naturally in certain bacteria. While the more classical methods of determining optical rotation usually can be used for characterizing the D-amino acids, occasionally, due to the small quantity of material available, the use of a specific enzyme preparation may be necessary for establishing the presence or the amount of these substances. (See Lipmann, et al., 1940, and Lipmann, et al., 1941.) For this purpose the use of simple aqueous extracts of acetone dried kidney powder (Krebs, 1935b) is usually satisfactory. Should a pure reconstituted flavin-adenine-dinucleotide-protein system be required, the resolution and reconstitution technique of Warburg and Christian (1938) can be employed. The specificity of the two preparations is the same (Klein and Handler, 1941). For the details of preparation and procedure, the reader is referred to the original papers.

L-AMINO ACIDS

The preparation of potent L-amino acid decarboxylases from bacteria has been reported by Gale (1946). These decarboxylases are particularly suitable for the manometric estimation of certain L-amino acids. Table XXX summarizes the information available on the specific amino acid decarboxylases and the conditions used for the estimation of the different amino acids. With some strains of S. faecalis, phenylalanine may be decarboxylated (McGilvery and Cohen, 1948). Krebs (1948) has recently reported that washed suspensions of Clostridium welchii, strain SR 12, not only specifically decarboxylate glutamic acid, but also contain a specific glutaminase. This preparation can therefore be used to determine both glutamic acid and glutamine by merely measuring the CO_2 produced in the case of the former and the NH_3 plus CO_2 produced in the case of the latter. Of interest is the statement by Krebs (1948) that stock suspensions of Clostridium welchii in 0.2 M acetate buffer (pH 4.9) are active for about one month. (Compare with Gale, 1947, Table XXX.)

TABLE XXX

Specific Amino-Acid Decarboxylase Preparations for Estimation of Amino-Acids (Gale, 1947)

Enzyme	Organism (N.C.T.C. no.*)	Conditions of culture			Specific preparation	Activity Maintained	Manometric estimation	
		Medium	Temp.	Time (hr.)			Buffer	CO_2 evolution as % theory
L-Lysine decarboxylase	Bacterium cadaveris (6578)	3% casein digest 2% glucose (1 L. medium = 50 estimations)	25°	24	Acetone (5 vol.)-ether-dried powder, kept 3 days at 0° before use Use ca. 10 mg. per test	4-6 weeks in desiccator	M/5 phosphate, pH 6.0 +acid-tip	92 98
L-Arginine decarboxylase	Escherichia coli (7020)	3% casein digest 2% glucose (1 L. medium = 40 estimations)	25°	24	Acetone (5 vol.)-ether-dried powder Use c a. 10 mg. per test	4-6 weeks in desiccator	M/5 phosphate-citrate pH 5.2	95
L-Histidine decarboxylase	Clostridium welchii, B.W. 21 (6785)	3% casein digest 2% glucose Heart muscle (1 L. medium = 20 estimations)	37°	16	Acetone (3 vol. for 30 min.)-ether-dried powder Use c a. 30 mg. per test	2-3 months in desiccator	M/5 acetate, pH 4.5	96
L-Ornithine decarboxylase	Clostridium septicum, PIII (547)	3% casein digest 2% glucose Heart muscle (1 L. medium = 20 estimations)	37°	16	Washed suspension of organism 20-30 mg. dry weight cells/ml. Use 0.5 ml. per test	24 hours	M/5 phosphate-citrate, pH 5.5 +acid-tip	92 98
L-Tyrosine decarboxylase	Streptococcus faecalis (6783)	3% casein digest 1% glucose 0.1% marmite (1 L. medium = 50 estimations)	37°	16	Acetone (5 vol.)-ether-dried powder Use c a. 10 mg. per test	2-6 weeks in desiccator	M/5 phosphate-citrate, pH 5.5	96
L-Glutamic acid decarboxylase	Clostridium welchii, SR 12 (6784)	3% casein digest 2% glucose Heart muscle Hydrogen (1 L. medium = 30 estimations)	37°	12	Washed suspension of organism 20-30 mg. dry weight cells/ml. Use 0.5 ml. per test	48 hours in H_2	M/5 acetate, pH 4.5	98

*National Collection Type Cultures, England.

PEPTIDASE ACTIVITY

Zeller and Maritz (1945) have reported that peptidase activity can be readily measured by the use of ophio-L-amino acid oxidase (Zeller and Maritz, 1944). The latter is a potent enzyme present in snake venoms and tissues. By adding an ophio-L-amino acid oxidase preparation to a peptidase-peptide system the liberated amino acid is rapidly oxidized and the O_2-consumption can be measured manometrically. The ophio-L-amino acid oxidase does not attack peptides. The rates of amino acid oxidation for Vipera aspis venom are given in Table XXXI and can be used as a guide in the choice of peptides suitable for this system.

TABLE XXXI

Amino Acid	Q_{O_2}	Amino Acid	Q_{O_2}
Phenylalanine	780	Diiodotyrosine	135
Leucine	612	Nitrotyrosine	129
Tryptophane	540	Histidine	78
Tyrosine	402	Valine	71
Methionine	390	Cystine	42
Dihydroxyphenylalanine	294	Alanine	30
Dibromtyrosine	216	Arginine	26
Isoleucine	162	Cysteine	22

DIPHOSPHO-PYRIDINE-NUCLEOTIDE (D.P.N., COENZYME I, COZYMASE)

Two manometric methods have been used in estimating D. P. N. The first method is that reported from von Euler's (1936) laboratory and is based on the fact that the rate of yeast fermentation is dependent, within certain limits, on the D. P. N. concentration. More recently, Axelrod and Elvehjem (1939) have reported on the details of this technique as applied to animal tissues. A second method is that proposed by Jandorf, Klemperer and Hastings (1941). The principle of this method is based on the enzymatic conversion of hexosediphosphate into phosphoglyceric and glycero-phosphoric acids. The rate of this conversion is dependent within certain limits on the amount of D. P. N. present. The reaction is carried out in a CO_2 bicarbonate medium and the CO_2 formed due to the formation of the above acids is measured manometrically. The advantages claimed for this technique over the former are:

1) The reaction is dependent on fewer enzymes and in particular, does not require the presence of the unstable carboxylase system;
2) Fewer and more readily available components, which are more stable, are required:
3) The need for almost daily standardization of reagents is avoided.

However, in practice, the choice of one or the other method will be determined by the experience of the investigator, the availability of the required preparations, etc. While the writer has had no actual experience with either method for purposes of assay, it would seem that the latter method is in principle a more sound one. The reader is advised to refer to the original papers for details.

MANOMETRIC ESTIMATION OF ENZYME ACTIVITY

Since many enzymes catalyze reactions which directly or indirectly involve the production or utilization of a gas, manometric methods have been extensively used in both assaying for enzyme activity, and in studying the kinetics of a given reaction. For obvious reasons, manometric methods have been most extensively employed in the study and assay of respiratory and other oxidation enzymes. The features of the manometric techniques used for enzyme study are:

1) The need for only small amounts of enzyme preparation,
2) The accuracy and speed of the estimation, and
3) The ease with which one can study the effects of inhibitors, substrate concentration, pH, and other aspects of kinetics.

A detailed discussion of the preparation and manometric study of different enzyme systems is beyond the scope of this chapter. The reader is referred to Green (1940) and Sumner and Somers (1947) for compilations of methods and details.

Since the principles and practice of manometry have been discussed in this volume, this technique should be applicable to any enzyme system which catalyzes a reaction which directly or indirectly involves the uptake or the production of a gas.

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Chapter XV

METHODS FOR THE ANALYSIS OF PHOSPHORYLATED INTERMEDIATES

That phosphorylated carbohydrate intermediates and related compounds play indispensable roles in intermediary metabolism of animal (and certain other) tissues is now well established (Lipmann, 1941; Kalckar, 1941). In recent years a great variety of analytical techniques have become available for the measurement of the individual components of tissues concerned in phosphorylative glycolysis. But since the time of Lohmann (1930) there have been few attempts to provide methods for the accurate determination of all of the compounds involved in phosphorylative glycolysis on the same sample of tissue. Yet if one wishes to study phosphorylation in intact tissues a "distribution method" of some sort is necessary. The compounds in which one is interested in such studies include the following: inorganic phosphate (ortho), total phosphorus, adenosine-tri-phosphate (ATP), adenosine-di-phosphate (ADP), adenylic acid (AA), fructose-1-6-diphosphate (hexosediphosphate), glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, phosphoglyceric acids, phosphopyruvic acid, triose phosphates (phosphoglyceraldehyde and dihydroxyacetone phosphate), glycogen, lactic acid, and coenzymes. Contemporary concepts of the interrelations between these materials have been reviewed by Lipmann (1941), Kalckar (1941), Burk (1939), Barron (1943), Lardy and Elvehjem (1945), Lipmann (1946), Ochoa (1946), Sumner and Somers (1947), and others.

The method described below is capable of estimating each of the materials listed above on a relatively small sample of tissue (1-5 grams wet weight depending upon its phosphorus content; 5-25 mg. organic phosphorus) providing interfering substances are not present in great quantity. This depends upon the tissue one is using. The method as described is satisfactory for muscle, heart, brain, kidney, and with modifications, liver tissue of the rat, similar organs of the rabbit, for yeast and for many bacteria. It has been used, in part, successfully in the identification of some phosphorylated compounds in spinach leaves (Bonner and Wildman, 1946) and oat coleoptiles (Bonner, 1948). It is, however, not capable of identifying all of the phosphorylated compounds which occur in the algae (Chlorella) (Emerson, Stauffer and Umbreit, 1944), in the developing oat seedling (Albaum and Umbreit, 1943), in photosynthetic bacteria, and in the anaerobic bacteria (Clostridium). One may conclude that certain of the phosphorylated compounds in these latter tissues are different from those found in muscle and yeast. For the most part, however, especially for animal tissues the method is satisfactory.

It must be recognized that in new experimental circumstances or in other kinds of tissue, modification of the procedure may become necessary. It contains, as now designed, no provision for estimating the phosphogluconic or phospho-ribonic series of compounds which are not particularly suited to separation by barium salts. However the scheme of analysis provides a basis from which one can expand to meet new experimental needs.

The method consists, in brief, of an intermittent extraction of the "acid soluble" phosphorus with trichloracetic acid, a separation of this extract into three well-defined fractions, and the determination of the known components of these fractions by means of their characteristic properties. When this has been done, a balance is made to determine how much of the phosphorus has been accounted for in terms of known compounds. This serves to check on the accuracy of the determinations and may also serve to detect new phosphorylated compounds especially where "unaccounted for" phosphorus is to be further investigated.

METHODS OF EXTRACTION FROM TISSUE

The ease of extraction of these compounds varies greatly with the type of tissue. In animal tissues cold trichloroacetic acid will extract the phosphorus esters and lactic acid. It is advisable to use 10% trichloroacetic acid in the first extraction in order

to compensate for the dilution of the acid by the tissue; further extractions to remove the material soluble, but held in the tissue mechanically, can be carried out with 5% trichloroacetic acid. If animal tissue, muscle for example, is homogenized (Potter and Elvehjem, 1936; see Chapter 11) in the trichloroacetic acid, the extraction takes only a few minutes and further contact with the acid for several hours does not appreciably increase the acid extractable phosphorus. However, with bacterial cells, it is usually necessary to treat the cells with approximately their own wet volume of acetone (or employ some other means of breaking down the cell) before adequate extraction of the acid soluble phosphorus is obtained. Even under these circumstances it often takes from two to five extractions of several hours each to remove all of the acid extractable phosphorus. Such prolonged extractions must be made in the cold to avoid hydrolyzing some of the labile materials. As a general procedure one extracts the tissue, then re-extracts the residue, repeating this process until no appreciable amount of phosphorus is obtained in the last extract. In each case it is necessary to be sure that the extract obtained contains all of the acid soluble phosphorus.

In experiments with animal tissues, removal of the tissue under anaesthesia may result in rapid changes which cannot be prevented. The use of freezing techniques appears to be more satisfactory (Davenport and Davenport, 1928; Kerr, 1935). Freezing the whole animal in liquid air, dissecting the tissue while frozen, powdering it in the same condition, and adding the frozen powder to trichloracetic acid in a weighed tube, has been a satisfactory method of operation in experiments on rats. The tube is reweighed (giving the weight of tissue), mixed briefly in a loosely fitting homogenizer, and the tissue residue centrifuged out. If the freezing is done without anaesthesia, marked changes occur due to reaction of the tissues to sudden contact with the liquid air (muscle contraction, for example). Resting values for lactic acid, high ATP, etc., are obtained if the rats are given nembutal (5 mg./100 gm. intraperitoneally) and frozen as soon as they reach light surgical anaesthesia (2-4 minutes).

Dissection is carried out in a cold room (0° C.) with chisel and hammer. With practice, one can obtain the whole kidneys, whole brain, etc. Care must be taken with brain and muscle samples not to include any bone with the sample.

The tissue sample is thrown into a steel cylinder chilled with liquid air, hammered several times with a heavy piston, and the powder chilled with liquid air again. As soon as the air has just boiled off, the powder can be transferred to a paper sheet and into the trichloracetic acid without thawing until it is in the acid.

One must work rapidly from this point until the labile esters, especially phosphocreatine, have been determined.

FRACTIONATION OF THE EXTRACTS WITH BARIUM

Separation of the acid soluble phosphorus compounds into three fractions, two of which contain known esters, can be accomplished by the use of the solubilities of the barium salts at pH 8.2. For such a fractionation, it is advisable to employ material containing a total of 5-25 mg. of phosphorus and to keep the original volume of the extract down to 10-50 ml. One uses concentrated solutions of reagents for the main part of the neutralizations, etc., to prevent appreciable increase in the volume of the fractions. The pH is easily adjusted to 8.2 by the addition of 1/300 volume of 1% phenolphthalein and addition of KOH to a just discernible pink. It is quite important that the pH is at 8.2 throughout the precipitation and variations of 0.5 pH unit on either side will cause the fractions to be contaminated with compounds which would otherwise occur elsewhere. A large excess of barium is added in the form of barium acetate. For example, each milligram of total phosphorus present theoretically would require 8.2 mg. barium acetate or 0.033 ml. 25% barium acetate solution. Actually for each mg. of phosphorus 0.25 ml. 25% barium acetate is added.

After the addition of the barium acetate there are two procedures which may be followed depending upon the nature of the tissue. If the trichloroacetic acid has extracted a relatively large amount of polysaccharide from the tissues, the second procedure (B) is usually more suitable. Procedure A, however, is generally more convenient and is entirely satisfactory in most cases. These procedures are diagrammed in Fig. 56 and described as follows:

FIG. 56

Methods of Extraction and Fractionation

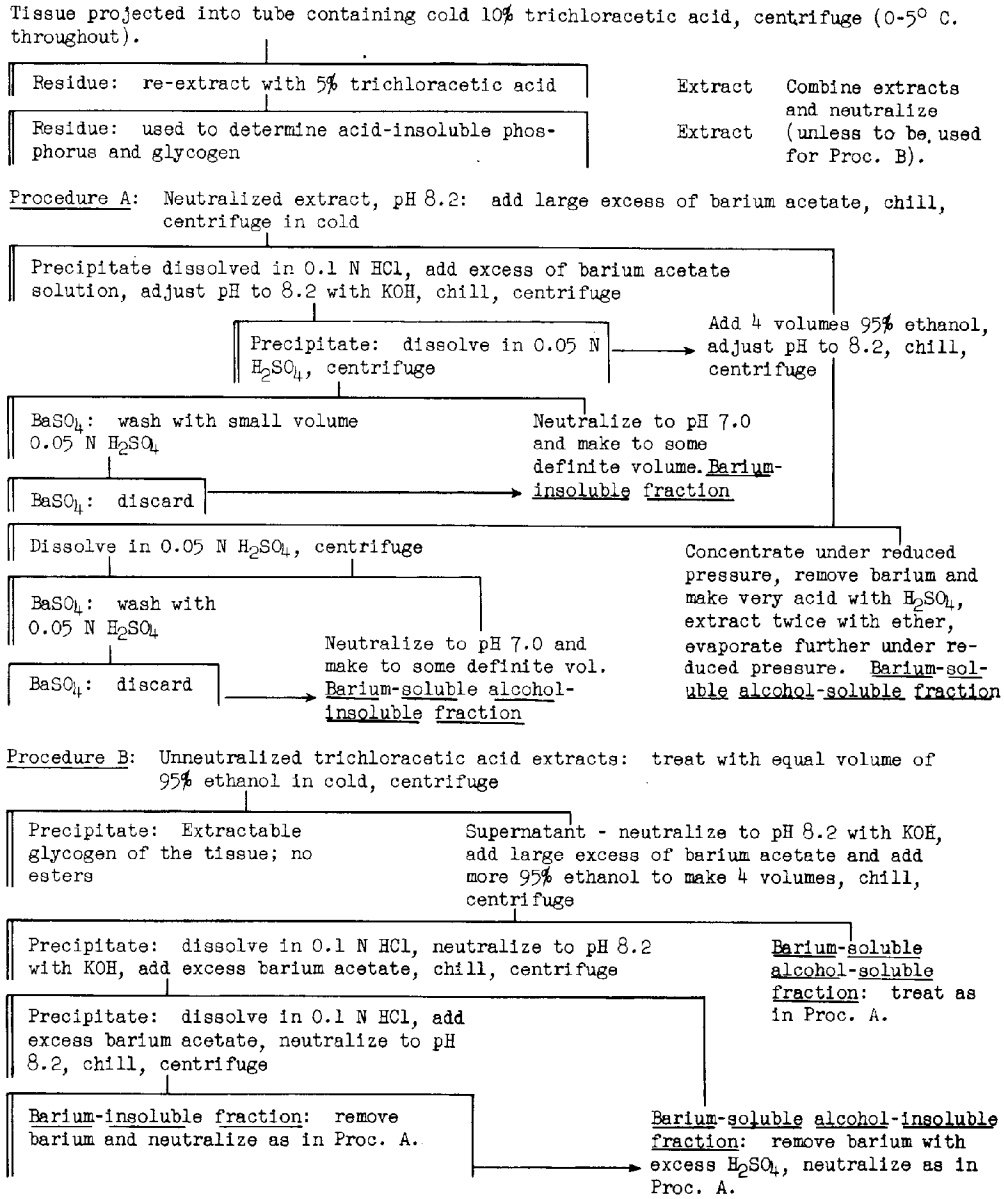

TABLE XXXII

Some Chemical Properties of the Phosphorylated Esters and Related Compounds

Fraction	Compound	Method of Determination	% Hydrolysis* in 1 N HCl at 100 C.		Reducing Values to Folin-Malmros Method
			7 Min.	180 Min.	
Barium-insoluble	ATP	△7 P, pentose	66	86	0
	ADP	△7 P, pentose	50	79	0
	Hexosediphosphate	Fructose	26.5	---	9.5
	3-Phosphoglyceric acid	Resistant to 3 hr. hydrolysis (corrected)	0	2	0
Barium-soluble alcohol-insoluble	Glucose-1-phosphate	Phosphorus and reducing sugar hydrolyzed in 7 min.	100	---	0 (66.4% after hydrolysis)**
	Glucose-6-phosphate	Reducing sugar	---	10.5	13.2
	Fructose-6-phosphate	Reducing sugar, fructose	---	74	31.6
	Phosphopyruvic acid	Phosphorus released by alkaline iodine	46	100	0
	Triosephosphate	Alkaline hydrolysis	46	100	---
	Adenylic acid	260 mμ absorption spectrum	---	58.7	0
	Phosphopyridine nucleotides	Nicotinic acid, reduced form - 340 mμ absorption spectrum	---	58.7	0
	Phosphocreatine	Inorganic phosphorus not precipitated with calcium	100	---	0
	Ribose-5-phosphate	Pentose	---	58.7	19.7
	Inosinic acid	Pentose, 248-290 mμ absorption spectrum, conversion to uric acid	---	58.7	0
Barium-soluble alcohol-soluble	1,2-Propanediol phosphate	Lead precipitation after removal of interfering factors	---	1.7	0
	Aminoethyl phosphate	Precipitation with inorganic phosphorus and uranium	---	---	0
Glycogen	Glycogen	Reduction after acid hydrolysis	----	100	0

*Hydrolysis rates of resistant esters are affected to some extent by the level of inorganic phosphorus present; those of easily hydrolyzable esters are not so affected.
**69.2 per cent theoretical, 66.4 per cent found.

Procedure A: The extract after the addition of barium (at pH 8.2) is chilled in a refrigerator for 15 minutes and the precipitate is centrifuged down. The precipitated material is not homogeneous, however, and contains, in addition to the "barium insoluble" compounds, some of the "barium soluble" material in the form of double salts (Cori and Cori, 1932). These can be removed by redissolving the precipitate and reprecipitating it as follows: The crude "barium insoluble" precipitate is dissolved in 0.1 N HCl, a drop of barium acetate solution added, and the pH adjusted to 8.2 with KOH. The suspension is chilled 15 minutes and centrifuged. The supernatant contains the "barium soluble" materials which had been precipitated with the "barium insoluble" compounds and is added to the original "barium soluble" fraction. The precipitate contains the "barium insoluble" materials. It is made acid with HCl, the barium removed by the addition of a slight excess of H_2SO_4 and the barium sulfate removed by centrifugation. The barium sulfate is washed with water by centrifugation and the washings added to the "barium insoluble" fraction. This is neutralized, made to a definite volume, and held for analysis. The compounds contained in it (see Table XXXII) are definitely "barium insoluble" (at pH 8.2) and further reprecipitation usually entails no loss.

The "barium soluble" fraction (including the soluble materials from the reprecipitation of the "barium insoluble" portion) is treated (pH 8.2) with 4 volumes of 95% ethyl alcohol, chilled for one-half hour, and the precipitate removed by centrifuging. It is important that it be cold since if the precipitate is taken at room temperatures the coenzymes are likely to be lost (Warburg and Christian, 1936). The precipitate is treated with HCl and a slight excess of H_2SO_4 to remove the barium as the sulfate, the $BaSO_4$ removed by centrifugation, and washed with N/100 HCl. The supernatants and washings are neutralized, made to a definite volume, and held for analysis. This constitutes the "barium soluble-alcohol precipitable" fraction (see Table XXXII).

The "barium soluble-alcohol soluble" portion is concentrated under reduced pressure, at 30-35° C. to small volume and held for analysis. The compounds present in this fraction are not known, with this exception: propanediol phosphate has been found (LePage, 1948b). In most tissues this fraction contains only a small proportion of the phosphorus, but in some tissues, especially plants, it may comprise as much as 30-40% of the acid-soluble phosphorus.

Procedure B: This is essentially similar to procedure A except that the "barium insoluble" and "barium soluble" fractions are precipitated together by the addition of alcohol in the first step. This tends either to remove, or to dehydrate, interfering polysaccharide materials (starches or bacterial gums) which sometimes act as protective colloids and prevent a clear separation of the compounds by procedure A. The diagram (Fig. 56) is self-explanatory. The best index as to when this method should be used is the inorganic phosphorus content of the "barium soluble" fraction. If it is zero by procedure A, the separation is probably satisfactory; if inorganic phosphorus appears in any quantity in any fraction except the "barium insoluble", procedure B should be used. There is an additional modification necessary for application to rat liver which will be described later.

BASIC METHODS FOR THE ANALYSIS OF THE FRACTIONS

The fractions obtained by the procedures outlined above are analyzed for the components known to occur in them by means of certain characteristic properties of these compounds. This entails the use of a series of micromethods. The ones we have employed are listed below. Obviously these can be modified and other procedures substituted at the convenience of the investigator. For all the methods described below we have employed the Evelyn (1936) photoelectric colorimeter using slightly smaller standardized tubes (150 by 19 mm.) with an adapter so that they would fit the instrument. If the usual size Evelyn tube is used, the same quantity of reagents, etc., may be employed but the range of the methods will be slightly less. In standardizing these methods we first set up standard curves using pure compounds. All methods follow Beer's law over the range indicated and are not subject to appreciable fluctuation. Each series of analyses includes a standard from which the contents of the samples are calculated and this standard must agree with the standard curve within the limits of precision of the method. The precision quoted is the standard deviation from the mean of a series of duplicates at approximately the center of the range given.

Inorganic (ortho) phosphorus, including phosphocreatine: This is determined after the method of Fiske and Subbarow (1925) as follows: The sample is mixed in a colorimeter tube with 0.4 ml. 10 N H_2SO_4, 0.8 ml. 2.5% ammonium molybdate, and 0.4 ml. of Fiske-Subbarow reducing agent (materials added in the order named), made to 10 ml. with distilled water. The color is developed at room temperature for 10 minutes. The 660 mμ filter is used. The range is 4-40 micrograms phosphate phosphorus with a precision of ± 0.2 micrograms.

In samples containing inorganic phosphate but no phosphocreatine, ten minute color development is sufficient. When phosphocreatine is to be determined as well, the sample should be incubated with the acid and the molybdate for twenty minutes before the addition of the reducing agent, to permit the hydrolysis of the phosphocreatine.

N.B. Precautions should be taken to exclude contamination of the extracts or reagents with silica from homogenizers or from alkaline reagents stored in soft-glass containers. With the concentrations of acid employed in the determination, silica will react as inorganic phosphorus. Some detergents used in washing glassware interfere with the method.

Reagent: Fiske and Subbarow (1925) reducing reagent: Grind 0.5 grams of 1-amino-2-napthol-4-sulfonic acid in 15% $NaHSO_3$, make to a volume of 195 ml. with 15% $NaHSO_3$ and add 5 ml. 20% Na_2SO_3. Warm until the materials are all in solution. This reagent is stable for several weeks if kept well stoppered to prevent loss of SO_2 and protected from strong light.

The value obtained by this method is a measure of both the ortho-phosphate and the phosphocreatine phosphorus since the latter is very rapidly decomposed by the molybdate reagent. Phosphocreatine is not found in yeast, plant, or bacterial tissues so far studied, hence in these tissues the method above measures inorganic phosphorus. In animal tissues, however, the apparent inorganic phosphorus is composed of two parts, the "true" inorganic phosphorus and the phosphocreatine phosphorus. These are determined as follows:

"True" inorganic phosphorus: The method is adapted from that of Fiske and Subbarow (1929). To the neutralized sample add one-fifth of its volume of 10% $CaCl_2$ saturated with $Ca(OH)_2$ at pH 8.8. Let stand at room temperature for 10 minutes. Centrifuge the precipitate and wash with a small volume of water containing 20% of the $CaCl_2$ reagent. The "true" inorganic phosphorus is thus precipitated; the phosphocreatine remains in solution. The washed precipitate is dissolved in dilute HCl and inorganic phosphorus determined on it as described above (calcium not removed). This value is the "true" inorganic phosphorus of the tissue. According to Stone (1943) calcium ATP is hydrolyzed by calcium hydroxide. In the method described above the values for both "true" inorganic and phosphocreatine are identical when the calcium precipitation is conducted at 0° C. or at 25° C. indicating that ATP is not measurably hydrolyzed during the time required (10 min.) for this precipitation.

Phosphocreatine: This is the difference between the apparent inorganic phosphorus and the "true" inorganic phosphorus, i.e., the phosphorus determined as "inorganic" by the method but which is not precipitable by calcium at pH 8.8. Since phosphocreatine is very labile in acid solution one must determine phosphocreatine as soon as possible after the extraction and keep all extracts cold until the aliquot for phosphocreatine has been neutralized. In trichloracetic acid phosphocreatine will be approximately 25% hydrolyzed in one hour at 15° C. A convenient means of avoiding use of a cold room is to keep the tubes immersed in a beaker of crushed ice until the phosphocreatine analyses are completed.

Total phosphorus: The sample is digested with 0.40 ml. of 10 N H_2SO_4 in an oven or sand bath for 30-60 minutes at 130-160° C., removed, partially cooled (this can be carried out in the pyrex colorimeter tubes) and 1-2 drops of 30% hydrogen peroxide added. The tube is replaced in the oven for 15-20 minutes, then partially cooled, and 1 ml. of water added to the residue. This diluted sample is now heated at 100° C. for 10 minutes to decompose pyrophosphates. The cooling before additions prevents the acid from fuming; fuming is undesirable because some phosphorus may be lost. Now inorganic phosphorus is determined without further addition of acid.

Reducing sugar: This is patterned after the method of Folin and Malmros (1929). The sample is made to 1.60 ml. in a pyrex colorimeter tube. To it are added 0.80 ml. of 0.40%

$K_3Fe(CN)_6$ and 0.40 ml. of cyanide-carbonate reagent. The solutions are mixed, heated in a boiling water bath for 8 minutes, cooled 1-2 minutes, and 2.0 ml. of ferric iron reagent added. The volume is made to 10.0 ml. with distilled water and the color read against a reagent blank at 520 mμ. The range is 4-40 micrograms of glucose with a precision of ± 0.4 micrograms. It is necessary to have glass bubbles on the tops of the colorimeter tubes to minimize reoxidation by air.

Cyanide-carbonate reagent: Dissolve 8 grams of anhydrous Na_2CO_3 in 40-50 ml. of water, add 15 ml. of freshly prepared 1% NaCN and dilute to 500 ml.

Ferric iron reagent: This is prepared by dissolving 20 grams of gum ghatti in 1 liter of water, filtering and then adding a solution of 5 grams of $Fe_2(SO_4)_3$, 75 ml. of 85% H_3PO_4 and 100 ml. of water. After mixing, about 15 ml. of 1% $KMnO_4$ is slowly added to destroy reducing materials present in the gum ghatti, and the solution is allowed to stand several days before use. This reagent is stable indefinitely.

Fructose: This is determined after the method of Roe (1934). The sample is made to 2 ml. in a pyrex colorimeter tube. 2 ml. of 0.1% resorcinol in 95% ethyl alcohol and 6 ml. of 30% HCl are added, mixed, heated for 8 minutes in a water bath at 80° C. to develop the color, cooled, and read in the colorimeter with the 490 mμ filter. The range is 10-100 micrograms of fructose with a precision of ± 0.4 microgram.

It is necessary to have either pure fructose, pure fructose-6-phosphate, or pure fructose-1:6-diphosphate as a standard. Methods of preparing the fructose phosphates are described in the literature (DuBois and Potter, 1943; Neuberg and Lustig, 1942; Neuberg Lustig and Rothenberg, 1943). Fructose-1:6-diphosphate may be obtained from the Schwarz Laboratories, New York. The color obtained per micromole of hexose diphosphate is 52.5% (fructose-6-phosphate, 60.5%) that obtained per micromole of pure fructose. If fructose is used as a standard this must be taken into account (see determinations of hexose-diphosphate and fructose-6-phosphate). The resorcinol solution is stable for at least a month. As far as is known the method is specific for fructose in such extracts, although other keto compounds (perhaps ketogluconic acids, etc.) might conceivably interfere were they phosphorylated and able to enter these fractions.

Pentose: This is determined by the method of Meijbaum (1939). Dilute the sample to 3 ml. in a pyrex colorimeter tube; add 3 ml. of 1% orcinol in 0.1% $FeCl_3$ dissolved in concentrated HCl, and heat in a boiling water bath for 30 minutes. Cool and read in the colorimeter with a 660 mμ. filter. The range is 4-40 micrograms of pentose with a precision of ± 0.4 microgram. This method determines pentose in ATP, ADP, adenylic acid and coenzymes, as well as in the free form. A standard of xylose, arabinose, or ribose may be used, since all three give the same value (Schlenck, 1942). The 0.1% $FeCl_3$ in concentrated HCl can be kept as a stock solution, but the orcinol should be added just before the determination is made (10 mg./ml.). Orcinol as purchased is usually unsatisfactory; it should be recrystallized from benzene.

Nitrogen: The sample is digested in a pyrex tube in an oven or sand bath at 150-190° C. with 1.0 ml. of 5 N H_2SO_4 (containing 150 milligrams of copper selenite per liter) for 12 hours or more. Then 1-2 drops of 30% hydrogen peroxide is added and the tube heated over a microburner until white fumes develop. The sample is cooled, washed into a colorimeter tube with small portions of water, 1.20 ml. of 5.5 N KOH added, mixed, and 1.4 ml. of modified Nessler's solution added. The volume is adjusted to 10.0 ml. and the contents of the tube mixed. After 10 minutes the transmission is measured in the colorimeter with the 520 mμ. filter. The range is 30-100 micrograms. The precision (± 1.0 microgram) is not as great as with other analyses, so it is well to have duplicates or triplicates.

Modified Nessler's solution: This is prepared by grinding 5.0 grams of KI and 5-7 grams of HgI_2 together in a mortar with 50 ml. of water and making up to 500 ml. with water. There should be an excess of HgI_2. Allow the mixture to stand overnight, then filter off the excess HgI_2. To 500 ml. of this filtrate add 160 ml. of a 1.5% gum ghatti solution. Mix and dilute to 1500 ml. This reagent is stable indefinitely.

Nicotinic acid: This is patterned after the method of Bandier and Hald (1939). The sample is mixed with an equal volume of 2 N KOH and heated for 1 hour in a boiling water bath (to hydrolyze coenzymes and nicotinamide). On cooling the sample is neutralized to pH 7.0 with 2 N HCl and diluted to 4.5 ml. with 0.2 M phosphate buffer of pH 7.0. The sample is equilibrated in a water bath at 78-80° C., 0.50 ml. of cyanogen bromide reagent added and the incubation continued for 5 minutes. After the sample has cooled to room temperature, it is mixed with an equal volume (5.0 ml.) of photol reagent and allowed to stand for 1 hour, protected from direct light. Its transmission is then measured at 420 mu. The range is 5-40 micrograms with a precision of ± 1.0 microgram.

Cyanogen bromide reagent: Add 10% KCN solution to a saturated solution of bromine in water until the latter is just decolorized.

Photol reagent: A saturated solution of photol, monomethyl-p-aminophenol sulfate, is made by shaking 3-4 grams in 50 ml. of water.

Both reagents should be made up fresh within 1-2 hours of use.

Lactic acid: This method is patterned after that of Barker and Summerson (1941). The sample, containing 3-30 micrograms of lactic acid, is pipetted into a clean 16 x 150 mm. test tube, made to 4.5 ml. with water, and 0.5 ml. of 20% $CuSO_4 \cdot 5H_2O$ added. Approximately 0.5 grams of $Ca(OH)_2$ is added and dispersed by shaking. The copper-calcium hydroxide precipitate removes interfering materials. The precipitate is redispersed several times in the course of 30 minutes or more. After centrifugation, a 1.0 ml. aliquot of the supernatant is transferred to a clean tube, with care not to include any of the precipitate. Tube and sample are chilled in an ice bath, and 6.0 ml. of concentrated H_2SO_4 is added slowly from a pipette or burette, with vigorous shaking. This precaution, cooling and shaking, avoids localized heating which can cause irregular results (further oxidation of the acetaldehyde to acetic acid). An alternative method is to slowly layer the 1 ml. aliquote over the surface of 6 ml. cold sulfuric acid (the tubes remaining in the ice bath). Giving the tubes a quick shake will mix the sample and the sulfuric acid without appreciable heating. Tubes are heated in a boiling water bath for 5 minutes, cooled to below 30° C., and 1 drop each of 4% $CuSO_4 \cdot 5H_2O$ and p-hydroxydiphenyl reagent added. By using the same dropper each time, including the standards, one need not measure these reagents except by drops. The additions are immediately dispersed by shaking and the tubes incubated at 28-30° C. for 30 minutes or more with occasional shaking to redisperse the reagent. The tubes are then heated in a boiling water bath for 90 seconds, cooled, and the samples transferred to colorimeter tubes. Transmission is read against a reagent blank at 565 mμ. The range is 3-30 micrograms with a precision of ± 0.4 microgram.

A standard curve can be run with either pure zinc lactate or C.P. 85% lactic acid. With the latter, the acid is diluted to approximately 1 N, boiled to depolymerize, then titrated with standard alkali and phenolphthalein, and diluted appropriately.

The sulfuric acid used should be of reagent grade, kept free of metals, organic matter and nitrates. Traces of nitric acid renders it unfit for use in this method.

Para-hydroxydiphenyl reagent: Dissolve 1.5 gm. of p-hydroxydiphenyl in 100 ml. of 0.5% NaOH. It will keep indefinitely in a refrigerator, or for several months at room temperature in a dark bottle.

A blank should always be run through the complete procedure to make sure that no contamination has occured from glassware or reagents. Necessary precautions in this method include care that the apparatus is clean, since contact of fingers with the lip of a tube can add sufficient lactate to influence results seriously. Glassware cleaned in chromic acid should be rinsed and immersed in dilute alkali solution (0.2 N NaOH) for a few minutes before final rinsing to insure removal of chromium. As soon as the operator becomes convinced of the necessity of the precautions outlined, especially cleanliness of glassware and slow addition of acid to the sample in the cold, consistently accurate results are easily obtained.

Adenosine triphosphate (ATP) and adenosine diphosphate (ADP): Both of these compounds are quantitatively precipitated in the barium-insoluble fraction. If inorganic

phosphate is absent, ADP becomes much more soluble, but in the presence of inorganic phosphorus the solubility is only 4 micrograms per milliliter. Both ATP and ADP contain adenine, ribose and phosphorus. They are the only pentose compounds in this fraction. ATP has two easily hydrolyzable phosphates; ADP has one. Consequently both can be calculated by measurement of ribose, inorganic and easily hydrolyzable phosphorus (7 minutes, 1 N HCl, 100° C.). For example, a molar ratio of easily hydrolyzable phosphorus to ribose of 1.85 indicates that 85% of the ribose is present as ATP, 15% as ADP. A check on the ribose analysis of the fraction can be provided by measurement of the absorption of the solution at 260 mμ. in the spectrophotometer (see determination of adenylic acid, page 194). This absorption is due to the adenine component.

Fructose-1:6-diphosphate (HDP): In the presence of inorganic phosphate, this compound is quantitatively precipitated with barium. In the absence of inorganic phosphate, it is considerably more soluble (pH 8.2, excess barium; soluble to the extent of 2.8 mg./ml.). Since it is the only fructose ester in the fraction, it can be estimated by measurement of fructose. As it does not react as the theoretical amount of fructose (52.5%), fructose measured must be multiplied by the factor 3.60 to convert it to HDP present. If pure HDP is used as a standard, no correction is necessary. Since it is the only reducing compound present in the fraction* (9.5% that of an equal weight of glucose), the measurement can be checked by determining reducing sugar.

Little HDP is normally found in intact tissues. Consequently it is a small part of the phosphorus in the fraction. If any appreciable amount is found, the easily hydrolyzable phosphorus attributed to ATP and ADP needs to be corrected for a 26.5% hydrolysis of the HDP phosphorus.

Analyses made under special circumstances involving large amounts of HDP and relatively less inorganic phosphate, such as analyses of reaction mixtures for glycolytic studies in vitro, require a different approach. Here HDP is more soluble and can be precipitated quantitatively with barium provided 10% ethanol is present.

Phosphoglyceric acid: This compound (3-phosphoglyceric acid) is usually a measurable component of the tissue phosphorus. It is quantitatively precipitated with barium. Although a colorimetric method is available for its measurement (Rapoport, 1937), it has generally proved unsatisfactory owing to interference of other materials. The ester can be determined by making use of its resistance to acid hydrolysis. From the inorganic phosphorus after 3 hours of hydrolysis (1 N HCl, 100° C.) and the total phosphorus of the fraction, a figure can be computed for "resistant phosphorus". This includes the phosphoglyceric acid phosphorus, but must be corrected for unhydrolyzed phosphorus of ATP and ADP (41.3% of the stable phosphate). This test may be refined, if desired, by treating an aliquot of the barium-insoluble fraction with mercury in acid solution to precipitate the nucleotides (phosphoglyceric acid is soluble) and with magnesia mixture in alkaline solution to precipitate most of the inorganic phosphorus, then carrying out measurement of total and 3 hour hydrolyzed phosphorus. Resistant phosphorus is now all phosphoglyceric phosphorus.

Phosphocreatine: This is computed from the inorganic phosphorus determined on the original extract, and on the calcium precipitate, by difference. It is apparently an accurate representation in muscle, brain, heart, kidney and tumor, but questionable in the case of liver. It is not found in plants or microorganisms. The phosphocreatine appears in the barium-soluble alcohol insoluble fraction.

Glucose-1-phosphate: This ester is easily hydrolyzable in acid and can be computed by measurement on the barium-soluble alcohol-insoluble fraction of either inorganic phosphorus or reducing sugar before and after hydrolysis (7 minutes, 1 N HCl, 100° C.). The two values usually agree well, that from reducing sugar tending to be slightly higher owing to slight hydrolysis of other esters. If phosphopyruvic acid or triosephosphate is present, a correction must be applied to the easily hydrolyzable phosphorus for a 46% hydrolysis of these esters.

*Glutathione is soluble to the extent of more than 2 mg./ml. under these conditions and will not occur in this fraction.

Fructose-6-phosphate: This can be computed from the fructose analyses on the barium-soluble alcohol-insoluble fraction, since it is the only fructose ester present in the fraction. It responds only 60.5% of the theoretical to the fructose test, so that fructose analysis must be multiplied by the factor 2.39 to obtain fructose-6-phosphate, unless the pure ester is used as a standard, in which case no factor would be necessary. It responds, without hydrolysis, to the reducing sugar test to an extent equivalent to 31.6% of its weight of glucose. It is slowly hydrolyzed in acid, requiring 5 hours to hydrolyze 90% in 1 N HCl at 100° C.

Glucose-6-phosphate: This ester is usually a major constituent of the barium-soluble alcohol-insoluble fraction. It has a reducing value equivalent to 13.2% its weight of glucose and can be calculated from the reducing sugar measured on this fraction corrected for reduction due to fructose-6-phosphate and ribose phosphate not combined in nucleotides.

In certain specific circumstances errors may be caused in this measurement by glutathione and glucose. However, glutathione will not precipitate in this fraction unless it exceeds 1.2 mg/ml. in the original extract, which is not a usual physiological circumstance. If a tissue is hyperglycemic, as may be especially true for autolyzed liver for example, there is danger that the analysis for glucose-6-phosphate by this means will be high because of precipitation of part of the glucose with barium and alcohol. If this circumstance is met, it can be surmounted by taking an aliquot of the barium-soluble alcohol-insoluble fraction and reprecipitating it several times with barium and alcohol, redissolving in water each time, all in the same tube. By this procedure it was found that approximately 85% of the glucose was lost on each precipitation and as many as four such precipitations resulted in no measurable loss of glucose-6-phosphate (some of the other esters are not so well recovered). Reducing sugar measurements on this material, corrected for fructose-6-phosphate, are free from errors caused by interfering materials.

Phosphopyruvic acid: This compound, when present, occurs in the barium-soluble alcohol-insoluble fraction. It is best to determine it on the original extract first. Its measurement is based on its property of being hydrolyzed by alkaline iodine to yield inorganic phosphorus (Lohmann and Meyerhof, 1934). The determination is made by diluting the sample to 1.35 ml. in a colorimeter tube, adding 0.1 ml. of 2 N KOH and 0.05 ml. of 0.1 M iodine in KI, incubating for 15 minutes, then adding acid and discharging the excess iodine with sodium bisulfite. Inorganic phosphorus is measured and compared with that obtained without alkaline hydrolysis. The difference is computed as phosphopyruvic acid phosphorus. (Iodoform formation is ordinarily not sufficient to interfere.) This compound does not usually accumulate to a measurable extent in animal tissues.

Triose phosphates (dihydroxyacetone phosphate and 3-glyceraldehyde phosphate): These are determined by measuring alkali-labile phosphorus. (Lohmann and Meyerhof, 1934.) Phosphopyruvic acid is not hydrolyzed in this test. The sample is mixed in a colorimeter tube with an equal volume of 2 N KOH, incubated for 20 minutes at room temperature, neutralized, and inorganic phosphorus determined. The inorganic phosphorus hydrolyzed in this test on the barium-soluble alcohol-insoluble fraction is a measure of triosephosphate. Methods of differentiating the two have been described by Utter and Werkman (1941). These esters do not ordinarily accumulate to a measurable extent in animal tissues.

Adenylic acid: When the necessary equipment is available (Beckman quartz spectrophotometer), adenylic acid is most simply estimated on the barium-soluble alcohol-insoluble fraction, in which it occurs, by measurement of the absorption at 260 mμ. in the spectrophotometer. Measurement of pentose sugars gives preliminary indication of the concentration. An aliquot of the fraction is diluted in M/15 phosphate buffer at pH 7.0 and transmission read against a buffer blank. The range is approximately 2-20 micrograms of adenylic acid per ml. (required 3-4 ml.). A standard curve can be constructed with adenylic acid or adenosine.

When the aforementioned equipment is not available, a second method may be used which has been demonstrated to give the same results with animal tissues. This method makes use of the property of adenylic acid by which all the adenine can be hydrolyzed by 1 N HCl at 100° C. in 10 minutes without any appreciable hydrolysis of the ribose phosphate component. This hydrolysis is carried out on an aliquot of the barium-soluble alcohol-insoluble fraction, with the hydrolyzed material neutralized to pH 8.2 and the ribose phosphate precipi-

tated as before with barium and alcohol. The adenine remains in solution. The precipitate is dissolved, the barium removed, and nitrogen analyses are carried out on it and on the original fraction. The difference, i.e., nitrogen lost on hydrolysis, is the adenine nitrogen; hence adenylic acid can be calculated.

Both methods require a correction for any adenylic acid present as phosphopyridine nucleotides (coenzymes I and II - DPN and TPN). These are determined separately.

Coenzymes: The phosphopyridine nucleotide coenzymes (coenzyme I and II) are not completely recovered here (some go into the barium-soluble alcohol-soluble fraction). The amount of the two (sum) that is present can be determined by measurement of nicotinic acid, which is a component of both, and calculation from this analysis.

With a Beckman spectrophotometer or similar instrument, a second method is available -- measurement of absorption of the reduced coenzymes at 340 mμ. Because of the reproducibility of results with this instrument, the range can be carried down to 5 micrograms (computed as DPN, since there is usually several times more DPN than TPN) with a ± 5% error. It is more accurate when higher levels are used. For this assay, two 0.50 ml. aliquots of the barium-soluble alcohol-insoluble fraction (containing 5-175 micrograms of DPN) are pipetted into 13 x 100 mm. tubes, 0.40 ml. of freshly prepared 2% $NaHCO_3$ is added to each and 0.10 ml. of water to one, 0.10 ml. of a freshly prepared 3% sodium hydrosulfite in 1% $NaHCO_3$ to the other. Both are incubated for 20 minutes at room temperature, then 3 ml. of 1% $NaHCO_3$-1% Na_2CO_3 solution is added to each and a stream of air is passed through each for 5 minutes. Both solutions are used to measure transmission at 340 mμ. against a blank of the $NaHCO_3$-Na_2CO_3 buffer. Some absorption due to other materials is obtained in the unreduced sample. The difference is absorption due to reduced phosphopyridine nucleotides. The figures so obtained agree with nicotinic acid assays within limits of error.

Ribose phosphate: There is in some tissues an excess of ribose, measured in the barium-soluble alcohol-insoluble fraction, above and beyond that accounted for by adenylic acid and phosphopyridine nucleotides. This appears to be free ribose phosphate. It can be determined by difference, using the ribose, adenylic acid and phosphopyridine nucleotide analyses of the fraction. The significance of this component, whether it is present as a product of nucleotide metabolism or of oxidative hexose degradation, is not known.

Glycogen: Except for tissues like liver, cold trichloroacetic acid does not extract glycogen, and it can be measured on the tissue residue left after the esters are extracted. This tissue residue is mixed with 5 ml. of 30% KOH and heated in a boiling water bath until completely dissolved (10-30 minutes), then treated with 6 ml. of 95% ethanol while hot, cooled, and the glycogen precipitate centrifuged out. This is patterned after the method of Good et al. (1933). The supernatant is discarded and the precipitate hydrolyzed 2 hours in 1 N HCl at 100° C. The glycogen obtained from the trichloroacetic acid extract of liver is hydrolyzed also. The hydrolysate is neutralized and the reducing sugar measured with a copper reagent, such as that of Shaffer and Somogyi (1933). The factor 0.927 can be used to convert glucose measured to anhydrous glycogen. This procedure is satisfactory for liver, kidney, heart and muscle, but another feature must be added in the analysis of brain. For this, the tissue residue is dissolved in KOH and treated with alcohol as before. The precipitate obtained is suspended in a warm mixture of chloroform-methanol (20 volumes of $CHCl_3$ and 80 volumes of methanol) and centrifuged. This is repeated twice before the hydrolysis in acid. The washing removed cerebrosides which otherwise cause error by hydrolyzing in acid to yield reducing compounds (Kerr, 1936).

Aminoethyl phosphate: This compound is found in autolyzing tissues, probably from breakdown of sphingosine. If present, it appears in the barium-soluble alcohol-soluble fraction where it can be precipitated with uranium and inorganic phosphate provided the fraction is reduced to a small volume. It can be identified by elemental analysis.

Propanediol phosphate: This compound is present in the barium-soluble alcohol-soluble fraction of tissues. It appears to constitute 5% of the acid-soluble phosphorus of brain, 1-2% of that of liver, kidney and tumor. Solubility of the uranium salt is unaffected by inorganic phosphorus (exceeds 4 mg./ml. in solubility). It can be precipitated from this fraction when the volume is sufficiently reduced (solubility in the presence of excess basic lead acetate is 0.13 mg./ml.) by basic lead. It is highly resistant to alkaline or acid hydrolysis.

DISCUSSION

These methods and the properties of the compounds have been tested by the use of pure esters. In most instances they have also been confirmed by isolation of the compounds, from larger samples of tissue, in high yields (40-85%). For tabulated analyses of animal tissues made with these methods, the reader is referred to reports in the literature (Albaum et al., 1946; LePage, 1946a, 1946b, 1948a, 1948b and 1948c).

The in vivo level of the high energy phosphate reservoirs in a tissue always depends on the balance between synthesis and utilization. As has been pointed out (LePage, 1946b) it is possible to have high energy phosphorus reservoirs remain in a tissue because of failure or blocking of enzymes of utilization. It has been reported that brains infected with a virus had higher levels of ATP than did control brains (Kabat, 1944). In another study higher phosphocreatine levels were observed in failing hearts than were found in the functioning control hearts (Wollenberger, 1947). In both instances the explanation that enzymes of ATP utilization were failing more rapidly than enzymes of ATP synthesis is plausible. In the former case it is possible that the virus behaves in the same manner as an anesthetic, since no anesthetic was used on the controls (see LePage, 1946a).

The techniques described here provide a means of evaluating the in vivo mechanisms of ATP synthesis and also the mechanisms of ATP utilization, provided the correlated processes of function and ATP synthesis (glycolysis and respiration) can be simultaneously appraised.

G. A. LePage

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Chapter XVI

PREPARATION OF PHYSIOLOGICALLY IMPORTANT INTERMEDIATES AND METABOLITES

INTRODUCTION

In the course of study of tissue metabolism, certain materials are required which are not available from chemical supply houses. The preparation of some of these is described in this chapter. Whenever possible synthetic methods are given on the condition, however, that we ourselves have successfully employed these syntheses. Certain materials are obtainable only by isolation. For these, the principle followed is that the starting material should be as high as possible in the component to be isolated. Only isolations which we ourselves have successfully done are described. Some of the materials are available on the market but in rather limited amounts from few sources. In these cases we have referred to the sources from which we have obtained the material without reference to whether any more of the material may be available or whether other suppliers are able to fill the need.

The material contained in this chapter is concerned only with the following subjects:

1. The preparation of the principle intermediates of the "Meyerhof-Embden System".
2. The preparation of the principle intermediates of the Kreb's "Tri-carboxylic-acid Cycle".
3. The preparation of certain important electron transporting systems and cofactors.
4. The preparation of certain widely used suspending media.

INTERMEDIATES OF THE MEYERHOF-EMBDEN SYSTEM AND RELATED COMPOUNDS

These phosphorylated materials play a key role in tissue metabolism. Discussion of their role will be found throughout the literature but especially in the papers and reviews of Burk (1939), Cori (1942), Barron (1943), Lipmann (1941), Kalckar (1941), Meyerhof (1941, 1942), Potter (1944, 1945), Sumner and Somers (1947), and others. Methods of determining these materials are discussed in Chapter 15.

GLUCOSE-1-PHOSPHATE

The preparation of glucose-1-phosphate (as $C_6H_{11}O_5 \cdot O \cdot PO_3 \cdot 2H_2O$) from starch and inorganic phosphate in the presence of phosphorylase from potato tubers is described by Hanes (1940), Sumner and Somers (1944) and McCready and Hassid (1944). Although we have had no experience with the procedures given by the latter two groups of workers, both appear to offer the particular advantages of a considerable saving of time and ease of removal of the dextrins remaining in the original digest. Additional information on its preparation will be found in a paper by Cori, Colowick and Cori (1937) in which its synthesis is described.

This ester is now available commercially (e.g., Dougherty Chemicals, 87-34 134th St., Richmond Hill, N. Y.).

J. F. Stauffer

FRUCTOSE-1-PHOSPHATE

This compound is not ordinarily considered to be an intermediate in the Meyerhof-Embden system, but there is some evidence to show that it may act as an intermediate between this system and bacterial polysaccharides. The method of preparation and the properties of the ester have been described by Macleod and Robison (1933). Tanko and Robison

(1935) and Pany (1942). Bone phosphatase is allowed to act on hexosediphosphate until one phosphate group is removed. This is removed at either end of the hexose-di-phosphate molecule yielding 50% fructose-1-phosphate and 50% of a mixture of fructose-6-phosphate and glucose-6-phosphate (the latter arising because of the action of a phosphohexoisomerase present in the bone phosphatase). The inorganic phosphate released and any residual hexosediphosphate is removed in the barium insoluble fraction (see analytical methods, Chapter 15) and the mixture of monophosphates is removed in the barium soluble alcohol precipitable fraction. The glucose-6-phosphate is oxidized to phosphogluconic acid (which is removed in the barium insoluble fraction) by bromine, and the fructose phosphates remaining are separated by the fractional crystallization of their brucine salts. Fructose-1-phosphate is as labile as glucose-1-phosphate to acid and is highly levo-rotatory.

"EMBDEN'S ESTER"

This is an equilibrium mixture of glucose-6-phosphate and fructose-6-phosphate. Its preparation from yeast has been described in some detail by Warburg and Christian (1932) (especially p. 456 ff.), and a convenient preparation from fresh yeast has been reported by DuBois and Potter (1943). The "ester" is valuable as a source of "monophosphates" for enzyme studies and as a source of glucose-6-phosphate and phosphogluconic acid.

W. W. Umbreit

GLUCOSE-6-PHOSPHATE (ROBISON ESTER)

Previous methods of preparing glucose-6-phosphate for use in biological experiments have been based on the isolation of this ester from a crude mixture of hexose monophosphates obtained by yeast fermentation. It has also been prepared by allowing phosphoglucomutase to act on glucose-1-phosphate (Colowick and Sutherland, 1942). A non-biological method of synthesizing the ester as devised by Lardy and Fischer (1946) is given below.

1,2,3,4-Tetraacetyl-β-D-glucopyranose is prepared from 6-trityltetraacetyl-β-D-glucose according to the procedure of Helferich and Klein (1926) (see Reynolds and Evans (1942)) except that the crystallization is made from a concentrated chloroform solution (not syrup) by the slow addition of dibutyl ether. The first crop of crystalline tetraacetyl-β-D-glucopyranose corresponds to a yield of about 67% with a melting point of 124-127° C. This material can be phosphorylated directly or after recrystallizing from chloroform by the addition of dibutyl ether to give the pure substance, m.p. 128-129° C.

1,2,3,4-Tetraacetyl-6-diphenylphosphono-β-D-glucopyranose is prepared by adding 6.0 gm. of diphenylchlorophosphonate (Brigl and Müller, 1939) dropwise to a cooled solution of 7.1 gm. of 1,2,3,4-tetraacetyl-β-D-glucopyranose in 20 ml. of anhydrous pyridine with continuous shaking and cooling in an ice bath. The reaction begins at once, and within a few minutes a copious crystalline precipitate of pyridine hydrochloride appears. The mixture is kept in the ice bath for 15 minutes and then placed in a refrigerator at 10° C. overnight. A few drops of ice water are added to hydrolyze the excess of acid chloride and after one-half hour the product is separated by pouring slowly into 600 ml. of ice water under continuous stirring. When the precipitate becomes granular, it is filtered off and again stirred up in fresh ice water. The product is filtered off, washed with cold water, and dissolved in 100 ml. of chloroform. The chloroform solution is washed once with dilute HCl and two times with distilled water, dried with anhydrous sodium sulfate, and evaporated under reduced pressure to a syrup. The product is crystallized by careful addition of petroleum ether (b.p. 60-80° C.), swirling, and allowing to stand for several hours; the process may be hastened by seeding or scratching. The product is filtered with suction, washed with petroleum ether, and dried. The yield is about 10.9 gm. (92% of the theoretical). It melts at 64-66° C. And is of sufficient purity for subsequent use. The pure substance may be obtained by recrystallizing from isopropyl ether, or from acetone by the addition of water. Its m.p. is 68° C. It is soluble in chloroform, acetone, benzene and ethyl alcohol. It has an optical rotation of $[\alpha]_D^{22} = +16.5°$ (c = 1.37 in anhydrous pyridine).

$C_{26}H_{29}O_{13}P$ (580.5). Calculated C 53.8, H 5.04, P 5.34

1,2,3,4-Tetraacetyl-β-D-glucose-6-phosphoric acid is prepared by shaking a solution of 7.0 gm. of tetraacetyl-6-diphenylphosphono-β-D-glucopyranose in 70 ml. of anhydrous methanol (prepared according to Lund and Bjerrum, 1931) with 0.7 gm. of platinum oxide (Adams' catalyst) in an atmosphere of pure dry hydrogen at a pressure slightly greater than 1 atmosphere. When the reduction nears completion, the free acid begins to crystallize in fine needles. The absorption of hydrogen stops abruptly when the theoretical quantity (8 moles) has been consumed (this required from 2.5 to 4.5 hours in several runs). After warming to dissolve the product, the catalyst is removed by filtering or centrifuging. An equal volume of petroleum ether is added in portions to the filtrate and crystallization allowed to proceed during slow cooling. The crystals are filtered with suction, washed with petroleum ether, and dried in vacuo at room temperature. The yield obtained is about 3.6 gm. (65% of the theoretical). The product melts at 126-128° C., and contains the theoretical amount of organic phosphorus. When recrystallized from anhydrous methanol by slow addition of petroleum ether, the substance melts at 127-128° C. A second crop of crystals of the original purity may be obtained by evaporating the mother liquors to dryness under reduced pressure at a bath temperature of 25° C. and recrystallizing the product from methanol-petroleum ether. Analyses have indicated that the substance crystallized with 1 mole of methanol which could not be removed by heating in vacuo without causing further decomposition. The optical rotation is $[\alpha]_D^{25} = +17.4$ (c = 1 (of methyl alcoholate) in anhydrous pyridine); calculated for solvent-free compound

$C_{14}H_{21}O_{13}P$ (428.3) Calculated C 39.3, H 4.94, P 7.23

$C_{14}H_{21}O_{13}P \cdot CH_3OH$ (460.3) C 39.1, H 5.47, P 6.73

The potassium salt of glucose-6-phosphate is prepared as follows: To 3.3 gm. of tetraacetylglucose-6-phosphoric acid (from methanol, see above) partially dissolved in 75 ml. of cold anhydrous methanol, a sufficient quantity of potassium methoxide in anhydrous methanol (prepared by the cautious addition of clean potassium metal to anhydrous methanol; solutions of 1-2 N have been used) to neutralize the free acid groups is added dropwise with shaking. Complete solution is attained after the first few drops are added. Cleavage of the acetyl groups is initiated by the addition of a catalytic excess of 1.5 milliequivalents of potassium methoxide. The potassium salt of glucose-6-phosphate begins to separate at once. The cleavage is allowed to proceed at refrigeration temperature in a tightly stoppered flask overnight. The product is separated by centrifuging, washing four times with anhydrous methanol, once with each of the following methanol-ether mixtures: 80:20, 50:50, 20:80, and twice with anhydrous ethyl ether. After drying in vacuo at room temperature the yield obtain by following this procedure was 1.65 gm. (68.5% of theoretical). It is essential to use only anhydrous solvents and thoroughly dried equipment in order to obtain good yields. The potassium salt must be stored under anhydrous conditions. The optical rotation of the potassium salt was $[\alpha]_D^{24} = +21.2°$ (c = 1.3 in water).

$C_6H_{11}O_9PK_2$ (336.3) Calculated: C 21.4, H 3.29, P 9.21

From the combined mother liquor and methanol washings an additional quantity of glucose-6-phosphate can be obtained as the barium salt. The slightly turbid alcohol solutions are treated with an excess of $BaBr_2$ in anhydrous methanol. When the barium salt has settled, it is separated by centrifuging, washed with absolute alcohol, and finally with ether. This procedure has resulted in a yield of 0.6 gm. of the barium salt (21% of theoretical of the original starting material) after purification as described below; thus the combined yield of the potassium and barium salts was 89.5% of theoretical. To obtain all of the product as the barium salt, $BaBr_2$ may be added after the deacetylation by potassium methoxide is completed, and the barium salt again purified as described in the following paragraph.

The tetraacetylglucose-6-phosphoric acid may be deacetylated as follows: 0.5 gm. of tetraacetylglucose-6-phosphoric acid is dissolved in 35 ml. of 0.66 N HBr and the solution heated on the steam bath for 3 hours. After cooling, pulverized barium hydroxide is added to neutrality. The solution is filtered and 4 volumes of ethanol added. When the precipitate has settled, the supernatant liquor is decanted. The precipitate is washed in succes-

sion with 90% ethanol, absolute ethanol, 75% ethanol-25% ether, 25% ethanol-75% ether, and finally with dry ether. After drying in air, the barium glucose-6-phosphate is dissolved by extracting successively with 20, 10, and 5 ml. portions of distilled water. To the clear filtrate 4 volumes of ethanol are added and the product separated and dried as above. The barium salt (0.33 gm.) prepared by this procedure was free of inorganic phosphate and on the basis of its organic phosphorus content was 93% pure (yield 72% of theoretical). Its rotation (purity based on phosphorus analysis) was $[\alpha]_D^{24} = +17.9°$.

H. A. Lardy

FRUCTOSE-6-PHOSPHATE (NEUBERG ESTER)

This compound is prepared by the acid hydrolysis of hexosediphosphate. After hydrolysis, the inorganic phosphate and the residual hexosediphosphate are precipitable as the barium or calcium salts leaving fructose-6-phosphate in solution, from which it may be precipitated (as the barium or calcium salt) by the addition of four volumes of alcohol. Details of the method are described by Neuberg, Lustig and Rothenberg (1943).

FRUCTOSE-1-6-DIPHOSPHATE (HEXOSE-DIPHOSPHATE, HARDEN-YOUNG ESTER)

This substance is now available in the form of relatively inexpensive barium or calcium salts from the Schwarz Laboratories (202 East 44th St., New York City) and Nutritional Biochemicals Corp., hence no longer need be prepared by the individual investigator. While Lebedev extracts have been widely used in its preparation, Neuberg and Lustig (1942a) and DuBois and Potter (1943) have described methods using fresh yeast. Neuberg, Lustig and Rothenberg (1943) describe a simple and convenient method of preparing the pure compound (by means of its acid salts) and give a rather complete summary of its chemical properties.

PHOSPHOGLYCERIC ACID

Two compounds are of particular interest, i.e., d(-) 3-phosphoglyceric acid (Nilsson-Lohmann ester) and d(+) 2-phosphoglyceric acid (Kiessling ester). The synthesis of both has been described by Neuberg (1943). In tissues they are in equilibrium, with the 3-ester predominating. The preparation of the 3-ester from both fresh or dried yeast is relatively simple (Neuberg and Lustig, 1942b) and consists of allowing the fermentation to go on in the presence of fluoride and acetaldehyde. Further, the acid is readily prepared from almost any tissue. Inasmuch as it is the only compound resistant to acid hydrolysis in the barium insoluble fraction of yeast and animal tissues it may be readily isolated by subjecting the separated barium insoluble fraction to acid hydrolysis (1 N HCl, 100° C., 6 hours), precipitating the inorganic phosphate and unhydrolyzed phosphoglyceric acid with barium, removing the barium with sulfuric acid, the phosphate with magnesia mixture, and reprecipitating the pure phosphoglyceric acid with barium. (See Chapter 15.)

W. W. Umbreit

DETERMINATION OF PHOSPHOGLYCERIC ACID BY OPTICAL ROTATION IN THE PRESENCE OF MOLYBDATE

This method is so clearly described in the original publication (Meyerhof and Schulz, 1938) that the only reason for describing it here is to provide the details for those to whom the original journal may not be available. The method is far more specific than that of Rapoport (see page 193); the only interfering substances being other α-hydroxy acids and excessively large amounts of inorganic phosphorus. Inorganic phosphorus may be removed with magnesia mixture and the phosphoglyceric may be separated from most other acids by precipitation with lead acetate at a pH of about five.

The optical rotation of the acidified solution of phosphoglyceric acid is determined before and after the addition of 1/3 volume of 25% ammonium molybdate. For d(-) 3-phosphoglyceric acid in N HCl Meyerhof gives $[\alpha]_D^{20} = -13.2°$; with molybdate $[\alpha]_D^{20} = -745°$.

The $[\alpha]_D$ of the naturally occurring equilibrium mixture of d(-) 3-phosphoglyceric and d(+) 2-phosphoglyceric acids is according to Meyerhof -650° to -670° in the presence of molybdate.

Example: The specific rotation is defined as

$$[\alpha]_D^t = 100\ \alpha/e\ c$$

Where: α = observed angle of rotation.
e = length of tube in decimeters.
c = grams of material dissolved in 100 ml. solution.
t = temperature of the experiments.
D = wave length of light used (D line of sodium).

Solution of 0.907 mg. 3(-)phosphoglyceric per ml. used (pure ester).

(no molybdate) = 0; after addition of 1/3 volume of 25% ammonium molybdate = -0.45°; length of tube = one decimeter. (21° C.).

$$c = 100\ \alpha/e\ [\alpha]_D^t = 100 \times -0.45/1 \times -745 = 0.0604$$

Thus 0.0604 grams exist in 100 ml., or 0.604 mg. per ml. This had been diluted 1/3 by the addition of molybdate, hence original concentration found was 0.604 x 3/2 = 0.906 mg./ml. found; taken: 0.907 mg./ml. Specificity permits its use in extracts without extensive purification.

H. A. Lardy

ENOL-PHOSPHPYRUVIC ACID

The procedure employed is modified from that of Kiessling (1935).

Quinoline to be used in this procedure should be freed of water by treatment with CaO, and distillation under vacuum, or CaO separated and the quinoline distilled at ordinary atmospheric pressure. The pyruvic acid should be distilled under vacuum (5-8 mm., 35-40° C.) and the first third of the distillate discarded to avoid including water.

20 gm. of pyruvic acid is dissolved in 60 gm. of quinoline and the mixture put in a flask, equipped with an electric stirrer, so that very efficient mixing can be obtained. The flask is immersed in an oil bath at 70° C. and a solution of 115 gm. of phosphorus oxychloride in 120 gm. quinoline added slowly over a period of 20 minutes, with rapid stirring. Stirring is continued for 5-10 minutes to complete the reaction; then the flask is stoppered and chilled in ice. During the reaction time, make additions of quinoline to prevent the pH from going below 3.

The contents of the flask are gradually added to 265 ml. of cold 50% NaOH in a beaker immersed in ice. Chopped ice may be added to the solution to keep the temperature down. If necessary, add more NaOH to make the solution just alkaline to phenolphthalein. Separate off as much as possible of the quinoline. Add an excess of barium acetate (about 560 ml. of 2M) and 2 volumes of 95% EtOH (chilled). If necessary readjust the pH to be just alkaline to phenolphthalein. Centrifuge out the precipitate and suspend it in 600-700 ml. of 0.1 N HCl. Add 162 gm. of Na_2SO_4. Stir vigorously or mix in a Waring Blendor. Centrifuge and wash the precipitate. Treat supernatant and washings with 65 gm. NH_4Cl + 250 gm. $MgCl_2$, neutralize to a phenolphthalein color and mix in the Waring Blendor. Add a few ml. of concentrated NH_4OH and let the solution stand in the refrigerator ½ to 1 hour. Centrifuge and discard the precipitate. Neutralize to pH 4.5 with acetic acid, add barium acetate and centrifuge off any $BaSO_4$ formed. Add an excess of barium acetate (25-35 ml., 2M) and two volumes of 95% EtOH. Adjust to pH 8.0, chill, and centrifuge the precipitate. Take up this precipitate in 300 ml. 0.1 N HCl and treat with norite in the cold, mixing well and filtering off the norite. If the solution is not almost "water-white", the norite treatment should be repeated. The clear solution is adjusted to pH 8.0, an excess of barium acetate and two volumes of 95% EtOH are added. The resulting precipitate is

centrifuged in the cold, and washed successively with 95% EtOH and ether, and dried rapidly in vacuo over $CaCl_2$.

The product (1.5-2.5 gms.) may still contain some inorganic phosphorus. If sufficient inorganic phosphate is present to be objectionable, it can be removed by reprocessing, treating with H_2SO_4 to remove barium, precipitation of the inorganic phosphate with magnesia mixture and reprecipitation of the phospho-enol-pyruvate with barium and alcohol.

G. A. LePage

PHOSPHOCREATINE

This compound is most easily prepared synthetically. The synthesis of Zeile and Fawaz (1938) has been found to be reliable. This involves the direct phosphorylation of creatine with $POCl_3$.

Grind 10 grams of creatine hydrate (Eastman) thoroughly with 9 ml. of 17 N NaOH. After the mixture has been completely homogenized, dilute with 100 ml. water and centrifuge off any undissolved material. Cool the solution in an ice-bath and add, with stirring, 4 ml. of $POCl_3$ and 15 ml. of 17 N NaOH, in that order. Repeat these additions three more times adding ice-cubes to the mixture between additions, so that the final volume of $POCl_3$ added is 20 ml. and that of 17 N NaOH is 75 ml. The final volume of the reaction mixture should be about 450 ml. Allow to stand 20 minutes in an ice-bath after the last addition. The precipitate (which is Na_3PO_4) is filtered off through asbestos-glass wool. The precipitate is washed with a few ml. of water and the washings are added to the filtrate.

The filtrate is now carefully neutralized with cold concentrated HCl to a weak phenolphthalein red, while in an ice bath, with efficient stirring. From this point on the solution must not be allowed to become acid because phosphocreatine is stable only in a slightly alkaline solution. The solution is then concentrated to a volume of 150 ml. either by distillation in vacuo at 25° C. or by blowing clean, dry compressed air over the surface of the solution in a large evaporating dish. The creatine which has not reacted is then filtered off.

The volume of the filtrate is then carefully measured and the inorganic phosphate determined (see Chapter 15). Add the calculated amount of $MgCl_2$ and NH_4OH to precipitate all of the inorganic phosphate as $MgNH_4PO_4$. Add a few drops of $CaCl_2$ solution to the filtrate to remove the rest of the inorganic phosphate. Filter. Then add 40 grams of crystalline $CaCl_2$ and stir until it dissolves. Add 3 volumes of cold 95% alcohol (neutralized before use if necessary) with stirring and cooling. Let the mixture stand for 30 minutes in the cold. Centrifuge off the precipitate. Wash it with a small amount of cold water (to remove NaCl) and then with cold alcohol. Dry. This preparation can be used for enzyme studies although it contains considerable NaCl. It is however, easily obtained analytically pure by the treatment of Fiske and Subbarow (1929).

The preparation above is dissolved in water so that the solution contains 0.4 mg. total phosphorus per ml. Add an equal volume of filtered, saturated, $Ca(OH)_2$. The precipitate (inorganic phosphate and calcium carbonate) is filtered off. The filtrate is cooled to 0° C. with the least possible exposure to air and made just acid to brom-cresol-purple with dilute HCl. Three volumes of cold alcohol are added with stirring. After 30 minutes in the cold, the precipitate is filtered with suction, washed with alcohol and dried in air. The pure material has the composition: $C_4H_8O_5N_3PCa \cdot 4H_2O$ and should give 9.66% P on analysis. A second reprecipitation may be necessary. The yield is about 1-2 grams.

For use in enzyme studies the calcium can be removed by precipitation as the oxalate, the carbonate, etc. In all manipulations it must be kept in mind that the compound is extremely labile in acid solution (25% decomposition per hour at 20° C.) but is quite stable in alkaline solution. Its determination in tissue extracts is described in Chapter 15.

A. L. Lehninger

ADENOSINE-TRI-PHOSPHATE

It was found in this laboratory (Dubois, Albaum and Potter, 1943) that magnesium anaesthesia minimizes the breakdown of ATP which ordinarily occurs when the animals are killed by decapitation without anesthesia. The magnesium solution was made to contain 25% $MgSO_4$ (51% $MgSO_4 \cdot 7H_2O$), and was injected intraperitoneally. By giving a series of small injections the animal can be completely paralyzed and finally anaesthetized without respiratory failure, with the muscles remaining completely relaxed, and with no cyanosis. It is advisable to give about 250 mg. per kilogram followed by 125 mg. per kilogram every 10 minutes until complete anaesthesia is attained. The animal can usually be killed about ½ hour after the first injection. The muscles are removed from the back and the hind legs and cooled in cracked ice until all have been removed and trimmed. The trimmed muscles are then weighed (about 500 grams) and ground in the cold room in the presence of an equal volume of ice-cold 10% trichloracetic acid in a Waring Blendor. The precipitated muscle proteins and fiber masses are separated from the filtrate by squeezing through cheese cloth, and the residue is re-extracted in the Waring Blendor with an equal volume of cold 5% trichloracetic acid, and again strained through cloth. The combined extracts are filtered through a Buchner filter and brought to pH 6.8 (bromthymol blue indicator) with 10 N NaOH. The remainder of the procedure is essentially that of Needham. The dibarium salt of the adenosine triphosphate, along with any adenosine diphosphate, hexosediphosphate, phosphoglyceric acid and inorganic phosphate, is precipitated by addition of an excess of barium acetate (3.0 ml. of 2 M $Ba(OAc)_2$ per 100 gm. muscle will assure an excess). The suspension is chilled in the refrigerator one-half hour, then the mixed barium salts are centrifuged and the supernatant discarded. The precipitate is dissolved in 0.2 N HNO_3 (30-50 ml. per 100 gm. muscle used). A small insoluble residue should be filtered or centrifuged off. The solution is treated with a mercuric nitrate solution (Lohmann's Reagent: 100 gm. $Hg(NO_3)_2 \cdot 8H_2O$ + 25 ml. H_2O conc. HNO_3; add the HNO_3 to the salt before the water), using 0.6 - 1.0 ml. per 100 gms. muscle used. After chilling in the refrigerator (15 min.), the precipitated nucleotides are centrifuged out and suspended in a small volume of water. Hydrogen sulfide is passed in to decompose the mercury salts, and the resulting HgS centrifuged, washed with acidified water, and discarded. The combined supernatant and washings are aerated until the excess of H_2S has all been removed. The solution is then neutralized to pH 6.8 and an excess of barium acetate added (1.0 ml. of 2 M $Ba(OAc)_2$ per 100 gm. of muscle will assure an excess here). The suspension is chilled one-half hour in the refrigerator, and the precipitate centrifuged. Successive washes of the precipitate are carried out with: (1) 1% $Ba(OAc)_2$ at pH 6.8 (6-8 times the volume of the wet precipitate); (2) 50% EtOH; (3) 75% EtOH; (4) 95% EtOH; (5) diethyl ether. The precipitate is dried in a desiccator over calcium chloride, preferably under vacuum.

Additional manipulations can be used to obtain a purer product. These involve the following: after the final barium ATP precipitate has been obtained, as above, before washing with alcohol and ether, one can dissolve it in 0.2 N HNO_3 and repeat the precipitation as mercury salt and conversion back to the barium salt. This gives a purer material. After centrifuging and even filtering off the HgS precipitate, one often finds a faint trace of HgS suspended in the solution. This contaminates the final product, and is undesirable. It can be removed before precipitating the Ba-ATP, while the solution is still acid, by addition to the solution of a small amount of $Ba(OAc)_2$ and 3-5 ml. of 0.1 N H_2SO_4 and centrifugation of the small $BaSO_4$ precipitate formed. This precipitate carries down with it the last traces of HgS.

Sodium and barium salts and the free acid are now available for purchase (Armour and Co.; E. Bischott, Ivoryton, Conn.; Rohm and Haas, Philadelphia; Sigma Chemical Co., 4648 Easton Ave., St. Louis 13; Schwarz Laboratories and others). However, some of these preparations contain not only varying amounts of ADP and adenylic acid, but also heavy metal contaminants, which render the materials somewhat toxic for enzymatic use. Usually the contaminated samples may be rendered usable by employing the additional purification procedure mentioned in the previous paragraph.

Yields obtained from rabbits anaesthetized with $MgSO_4$ generally exceed 3.5 grams of the barium salt per kilogram of muscle. The material reprocessed as described contains 0.1-0.2% inorganic phosphorus, and is 98-99% pure calculated as the tetrahydrate (Ba_2 ATP $\cdot 4H_2O$). Two-thirds of the organic phosphorus is released as inorganic phosphorus on hydrolysis 7 minutes at 100° C. in 1 N HCl.

ADENOSINE-DI-PHOSPHATE

The method we have employed is modified from that of Lohmann and Shuster (1935), as follows:

A live lobster was cut in half, the tail muscles removed and 32 gm. of muscle weighed out. This was cut into small strips with a scissors and suspended in 300 ml. of chilled 0.45% KCl. After gentle agitation for 15 minutes, the muscle was filtered off and the supernatant discarded. The washing with 300 ml. portions of chilled 0.45% KCl solution was repeated four times. The muscle was then suspended in 100 ml. of a solution containing 1.0 gm. of adenosine triphosphate at pH 7.0 and incubated for 20 minutes in another 100 ml. of 1% ATP. The muscle was filtered off and the easily (7 min., 1 N HCl, 100° C.) hydrolyzable phosphorus determined. This had decreased 52%.

The two solutions (freed of muscle) were mixed, adjusted to pH 6.8, and precipitated with an excess of 1 M barium acetate. After leaving it in the refrigerator one-half hour, the suspension was centrifuged and the supernatant discarded. The precipitate was dissolved in 0.2 N HNO_3 (solution kept cold) and treated with Lohmann's reagent (see preparation of ATP). The precipitate, after chilling for 15 minutes was centrifuged out and the supernatant discarded. The mercury precipitate was suspended in 150 ml. of water and decomposed with hydrogen sulphide. The HgS was centrifuged out, washed, and solution and washings aerated free of H_2S. Final traces of HgS, which tend to be colloidal, can be removed by the addition of barium, and a few ml. of 0.1 N H_2SO_4 (centrifuge out the $BaSO_4$). This carries down the last of the HgS. The solution was adjusted to pH 6.8, one volume of 95% EtOH and an excess of barium acetate added. This precipitates the pure barium salt of adenosine diphosphate. (Omitting the alcohol greatly reduces the yield because BaADP is much more soluble in pure solution than when inorganic phosphates are present.) The precipitate was washed successively with 100 ml. portions of 50% EtOH (neutralized), 75% EtOH, 95% EtOH, and diethyl ether. It was then dried in a desiccator under vacuum, with $CaCl_2$.

Assuming the same water of crystallization as the ATP (4 H_2O), the product was 98% Ba·ADP·4 H_2O. The ratio of total organic phosphorus to easily hydrolyzable phosphorus was 2.03:1.00. Inorganic phosphorus was 0.31%. ADP is available from Sigma Chemical Co., St. Louis.

G. A. LePage

ADENYLIC ACID

Two types of adenylic acid are available. The muscle adenylic acid with the phosphate in the 5 position on the ribose has the structure present in animal, and most bacterial adenosine-tri-phosphate (ATP) (LePage and Umbreit, 1943), and is therefore the one to be employed with such tissues. It is obtainable from E. Bischoff, Ivoryton, Conn.; Sigma Chemical Co., 4648 Easton Ave., St. Louis, Mo.; Nutritional Biochemicals Corp., 8123 Jones Road, Cleveland, Ohio, and elsewhere.

Yeast adenylic acid containing the phosphate in the 3 position is available from the B. L. Lempke Co., 248 W. Broadway, New York, N. Y., Schwarz Laboratories, 202 East 44th St., New York, N. Y. and Nutritional Biochemical Corp., and may be readily isolated from yeast nucleic acid (Eastman Kodak Co., Rochester, N. Y.) by the method of Jones and Perkins (1925). So far as is known there is only one ATP in which this structure occurs (LePage and Umbreit, 1943). The two acids may be distinguished from one another by means of the hydrolysis rate of the ribose phosphates (LePage and Umbreit, 1943) and by means of the test described by Klimek and Parnas (1932).

The position of the phosphate on the ribose in the ATP isolated from plant tissue (Albaum et al, 1949) is not known.

ADENOSINE

This compound is available from the Schwarz Laboratories, Nutritional Biochemicals Corp. and elsewhere.

ADENINE

This material is available from most chemical supply houses including the Eastman Kodak Co., Chemical Sales Division, Rochester, N. Y.

INOSINE TRIPHOSPHATE

This compound is best made by deamination of adenosine triphosphate with nitrous acid according to Kleinzeller (1942).

The preparation of ATP has been described elsewhere (page 204) and a fairly pure preparation should be used. Two grams of Ba-ATP are dissolved in 0.1 N HCl, the Ba removed with Na_2SO_4, and the filtrate neutralized and brought to a volume of 32 ml. To this solution are added 4.52 gm. $CH_3COONa \cdot 3H_2O$, 10 ml. glacial acetic acid, and 20 ml. 60% $NaNO_2$. The pH of the solution should be about 4.0. After standing 5-6 hours at room temperature, cool with ice and add 2 N NaOH until the pH is between 6.0 and 6.5. Add 12 ml. 3N $BaCl_2$ and complete the precipitation of the Ba salt of ITP by adding 2 volumes of cold alcohol. Centrifuge off the precipitate and suspend it in 50 ml. H_2O. Add 3N HCl to the ice-cold suspension to bring the final concentration of HCl to 0.1 N. Shake the solution persistently until no more of the precipitate dissolves. Centrifuge off the precipitate and re-extract it with 20 ml. 0.1 N HCl. Add 1 gm. of urea to the combined centrifugates to destroy the remaining HNO_2. Keep at 0° C. for 2 hours. Then add 25 ml. 25% barium acetate and then a volume of alcohol equal to the total volume of solution plus barium acetate. Centrifuge off the precipitate, redissolve in 0.1 N HNO_3, and precipitate the ITP with Lohmann's reagent as is done in the preparation of ATP. The Hg compound is washed once with water, suspended in 50 ml. water and the Hg removed by passing H_2S through the solution. The HgS is removed by centrifuging, washed, and the combined centrifugates are aerated to remove H_2S. The solution is made 0.1 N in HCl and the Ba salt precipitated as above (excess Ba acetate plus one volume alcohol). Redissolve and reprecipitate. Wash twice with water, twice with 50% alcohol, twice with absolute alcohol and once with ether. Dry in vacuo over $CaCl_2$. The yield is approximately 800 milligrams. The purity of the compound can be established by methods used for ATP. The empirical formula for the product is $C_{10}H_{10}O_4N_4P_3Ba_2 \cdot 7H_2O$ calculated % 7'P = 6.86%, total P = 10.3%, N = 6.20%. Ratio 7'P/total P = 0.667. The product will be almost completely pure, by these standards.

For use in enzyme experiments, the compound is converted into the sodium salt in the same way as is ATP. It is determined in an identical manner, i.e., by the phosphorus liberated by 7 minute hydrolysis in 1.0 N HCl at 100° C.

INOSINE DIPHOSPHATE

This compound is prepared by enzymatic hydrolysis (by a myosin preparation) of inosine triphosphate. The methods have been outlined by Bailey (1942) and Kleinzeller (1942).

The myosin or lobster muscle extract is prepared as in the section dealing with the preparation of adenosine diphosphate (page 205). The incubation of inosine triphosphate with the extract is carried out exactly as described there. To isolate the product from 1 gm. of the Ba-ITP, the myosin is removed by acidifying the reaction medium to pH 5.0 and centrifuging off the precipitate. The cooled supernatant is brought to pH 6.0 with N NaOH and 2-3 ml. of 25% barium acetate added. The resulting precipitate is centrifuged off and discarded. The supernatant is brought to pH 7.0 and the IDP precipitated with excess 25% barium acetate and an equal volume of cold alcohol. The precipitate is separated and suspended in H_2O. 3N HNO_3 is added to make a final concentration of 0.1 N and excess barium acetate and an equal volume of alcohol added as before to precipitate the Ba-IDP. The compound is treated with Lohmann's reagent (see preparation of ITP, ATP) and after removal of the Hg with H_2S the filtrate is again adjusted to 0.1 N HNO_3, precipitated twice with barium acetate and alcohol as before. The product is washed with 50% alcohol until the washings are free of Ba (test with Na_2SO_4). It is then washed with 75%, then 95% alcohol, and ether. It is dried over $CaCl_2$ in vacuo. The yield is about 50-60% of theory. The product is very nearly pure. Calculated for $C_{10}H_{11}O_{11}N_4P_2Ba_{1.5} \cdot 6H_2O$; 7 min. P,4.20%; total P,8.39%; N,7.05%. Ratio 7 min. P to total P = 0.50.

The compound is converted to the soluble sodium salt by treatment with Na_2SO_4 in acid solution.

INOSINIC ACID

The method of Ostern (1932) has been found practical for the isolation of inosinic acid as the barium salt from fresh muscle tissue. One kilogram of fresh muscle (rabbit muscle has been used) is ground fine with a meat grinder and the ground tissue allowed to stand at room temperature for 3 hours to allow maximal enzymatic deamination of adenylic acid to inosinic acid. It is then mixed with a liter of water and slowly heated to boiling with constant stirring. Two ml. of glacial acetic acid are added to the mixture and it is boiled another three minutes. The mixture is then neutralized with NaOH and then made slightly acid with acetic acid, filtered through a fluted paper, and the residue pressed free of liquid. An excess of hot saturated $Ba(OH)_2$ solution is added to the filtrate to precipitate the inorganic phosphate and the mixture is allowed to stand for several hours. After testing for completeness of precipitation of inorganic phosphate by $Ba(OH)_2$, the supernatant is decanted and the residue centrifuged. The precipitate is washed with a few ml. of warm water and the washings combined with the supernatant. The combined solutions are neutralized with glacial acetic acid and saturated aqueous lead acetate solution added to precipitate the nucleotide. Do not add a great excess of the reagent. After standing several hours the precipitate is filtered off and washed. It is then suspended in 300 ml. water and decomposed with H_2S. The precipitate (PbS) is thoroughly washed with warm water saturated with H_2S to remove the considerable amount of nucleotide adsorbed on the PbS. Powdered $BaCO_3$ is added to the combined filtrate and washings in excess, the solution brought to a boil, filtered hot, and concentrated in vacuo at 40-50° C. to about 50 ml. The solution is filtered and further concentrated to a volume of about 10 ml. A white precipitate forms during the concentration. After the concentration is complete, the mixture is put at 0° C. for two days. The crystalline precipitate is filtered off cold and washed with a few ml. cold water. It is then dissolved in a minimum volume of boiling water, filtered hot, and allowed to crystallize at 0° C. The crystals are filtered off and washed with cold water. The yield is about 400-500 mg. pure barium inosinate. It is converted into the soluble sodium salt by removal of the Ba with Na_2SO_4.

A. L. Lehninger

PHOSPHOGLUCONIC ACID

This compound is readily prepared from either Embden's ester or glucose-6-phosphate by the method of Robison and King (1931). Using Embden's ester we have employed the following method:

Embden's ester (0.5 gm.), barium carbonate (0.6 gm.) and water (4 ml.) are ground together. Bromine (0.07 ml.) is added and the mixture stoppered and allowed to stand (30° C.) for 24 hours. The same quantity of bromine is again added and the mixture incubated for another 24 hour period. The excess bromine is removed by aeration, the mixture neutralized to pH 4 (to bring all of the barium carbonate into solution). The mixture is then fractionated according to the analytical procedure (Chapter 15), the barium insoluble fraction containing the phosphogluconic acid and inorganic phosphate. The barium is removed, the phosphate precipitated with magnesia mixture (see below), and the phosphogluconic acid precipitated with barium. Yield: 200 mg. (Ba salt). By redissolving and reprecipitating the barium salt (dissolve in acid, reprecipitate at pH 8.2) an analytically pure product is easily obtained.

ACETYL PHOSPHATE

The synthesis of this compound has been described in detail by Lipmann and Tuttle (1944).

PHOSPHORYLATING AGENTS

The preparation of a number of phophorylated intermediates has been substantially improved by the introduction of new phosphorylating agents. Most notable among these is diphenylchlorophosphate, whose preparation is described in detail by Baer (1949).

W. W. Umbreit

MAGNESIA MIXTURE

Preparation: Dissolve 55 gm. of $MgCl_2 \cdot 6H_2O$ and 100 gm. of NH_4Cl in 500-600 ml. of water. Add 100 ml. of 15 M NH_4OH and make up to a liter. If the resulting solution is turbid, it should be filtered.

Conditions: This reagent is used in many instances for removing inorganic (ortho) phosphate from solutions of phosphate esters, etc. The desired product is $MgNH_4PO_4 \cdot 6H_2O$. If conditions are not correctly adjusted, the precipitate obtained may be $Mg(OH)_2$ or $Mg_3(PO_4)_2$. The solution is made alkaline to phenolphthalein with NH_4OH, an excess of magnesia mixture added, and sufficient NH_4OH added to make the solution 1.5 M with respect to NH_4OH. If stability of the other materials permits (esters, etc.), the solution should be left 4 hours at room temperature to obtain quantitative precipitation of the inorganic phosphate as $MgNH_4PO_4 \cdot 6H_2O$.

Since the three phosphate radicals exist in equilibrium in solution, the amount of each will depend on the pH. Here one wishes to favor the ion HPO_4^{--}, which is accomplished by the use of NH_4OH. The function of the NH_4Cl is to prevent the precipitation of the $Mg(OH)_2$.

G. A. LePage

PRINCIPLE INTERMEDIATES IN THE KREBS' "TRICARBOXYLIC-ACID CYCLE"

After a long and somewhat controversial history, the Krebs' cycle has become a component part of our thinking with respect to animal metabolism. The subject has been very comprehensively reviewed by Krebs (1943). So far as is known the cycle as such does not occur, at least without modification, in molds or bacteria studied and there is indeed some doubt that, in spite of the fact that its component acids occur widely in plants, the cycle itself is in operation in these organisms. Integration of this scheme into the general metabolic picture of animal metabolism has been attempted by Potter and Elvehjem (1938) and Potter (1944). The compounds described below are the principle intermediates of the cycle. However, one cannot, of course, be sure that they are the actual intermediates, since rapid phosphorylation and dephosphorylation, for example, are entirely possible. At least these are the materials one puts into the reactions and these are the materials one again obtains. Analytical methods for these compounds are described in Chapters 13 and 14.

The other components of the cycle, e.g., citric acid, succinic acid, fumaric acid, and malic acid, are readily obtainable commercially. Brief remarks on the use of these are contained in Chapter 11.

CRYSTALLINE SODIUM PYRUVATE

Pyruvate solutions for use in tissue respiration experiments are usually prepared by suitable dilution and neutralization of pyruvic acid redistilled at reduced pressure and stored in a cold place. A more convenient means of preparing pyruvate solutions for use as a substrate is by weighing out crystalline sodium pyruvate. This salt may be prepared analytically pure from commercial pyruvic acid by the procedure of Robertson (1942).

Commercial pyruvic acid is dissolved in ten volumes of ethyl alcohol. This solution, while being rapidly stirred with a power stirrer, is slowly neutralized with a solution of

1 volume saturated NaOH in 10 volumes of ethyl alcohol at room temperature. Sodium pyruvate crystallizes out immediately. The crystalline material is filtered off, washed with alcohol and ether on the filter and dried in a vacuum dessicator. The yield is about 85% of theory. This material is sufficiently pure for most purposes. Analytically pure salt may be obtained by dissolving in a minimum amount of water and adding cold ethyl alcohol to 80% by volume.

The potassium salt may be prepared in the same manner with somewhat better yields, although recrystallization is sometimes difficult. The lithium salt is preferred by some workers.

ISOCITRIC ACID

In many metabolic experiments isocitric acid may be replaced by cis-aconitic acid, for the two are in equilibrium in many tissues. The synthesis of isocitric acid has been described by Pucher and Vickery (1946) and the isolation of the optically active compound from the leaves of Bryophyllum calycinum has been outlined by Pucher and co-workers (1948).

CIS-ACONITIC ACID

Trans-aconitic acid is readily synthesized by the method of Bruce (1937). It may be obtained from Chas. Pfizer & Co., 630 Flushing Avenue, Brooklyn, N. Y. Cis-aconitic acid may be prepared from it by the method of Malachowski and Maslowski (1928) as follows:

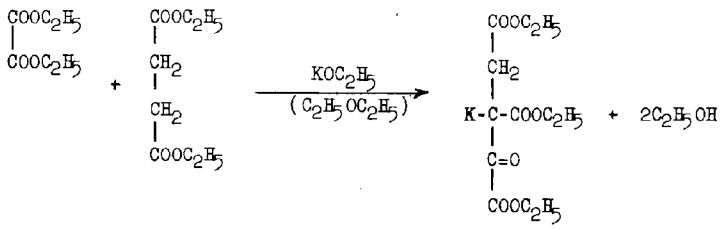
Recrystallize trans-aconitic acid from ether and concentrated HCl until the melting point is at least 182° C. Pulverize finely and mix with an equal weight of acetic anhydride and keep at room temperature for three days. The residue is filtered off, washed twice with a 1:3 mixture of acetylchloride and chloroform and once with a small quantity of chloroform. The solid material is trans-aconitic anhydride. The filtrate and washings are combined and evaporated at room temperature under reduced pressure until the acetic acid has been removed. When dry, extract with boiling benzene and filter hot. Crystals of cis-aconitic anhydride with ½ mole of benzene form on cooling. The benzene is lost when the product is dried and the anhydride melts at about 74°. Further purification can be accomplished by recrystallizing from benzene. The anhydride has been found to be stable for two years and immediately forms cis-aconitic acid in water.

H. A. Lardy

ALPHA-KETOGLUTARIC ACID

This compound may be synthesized according to the following directions, which are a modification of those proposed by Clutterbuck (1927): The directions are primarily those of Dr. D. J. O'Kane to whom we are indebted for them.

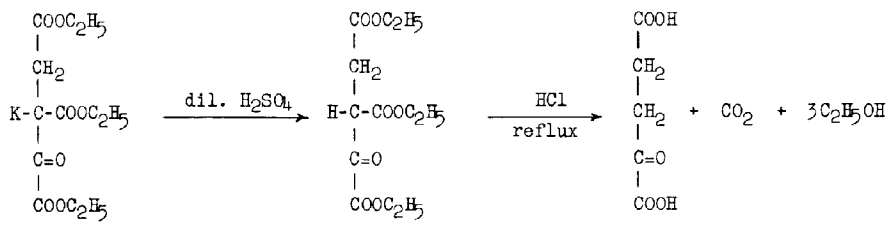
Potassium ethyl oxalsuccinate is prepared first; the type reaction is the acetoacetic condensation:



which is carried out as follows: To a mixture of 150 gm. of absolute ether (freshly distilled from sodium), and 40 gm. of absolute alcohol, add slowly 13.4 gm. of thinly sliced potassium. Warm the mixture with hot water under reflux until all the potassium has dis-

solved. Add 50 gm. of ethyl oxalate (Eastman). The solution turns from light yellow to deep yellow and a slight turbidity appears. After 10 minutes standing, add 59.5 gm. of ethyl succinate (Paragon), dropwise with shaking. A silky precipitate appears at the beginning of this addition, but disappears when about half of the ester has been added. Toward the end of the addition or shortly thereafter, crystallization begins, and the reaction mixture soon solidifies. After two hours standing, cut up the mass with a spatula, remove to a Buchner funnel, and press with a rubber dam. Cut the cake, wash once with ether, and press again with a rubber dam. Divide the cake as finely as possible and allow to air dry. The yield varies from 65-85% (64-84 gm.).

The α-ketoglutaric acid is now obtained by carrying out the following reactions:



Suspend the potassium ethyl oxalsuccinate in 250 ml. of water, bring to pH 2 with H_2SO_4, and extract with three 30 ml. portions of ether. Dry the combined extracts over Na_2SO_4 and remove the ether by distillation. The yield is about 85% on the basis of the potassium salt. Reflux the oxalsuccinic ester for 30 minutes with six times its weight of concentrated HCl diluted with one part of water. The two hour period recommended by Clutterbuck was found to be unnecessary since shorter periods gave better yields and a lighter colored product. Evaporate the solution to dryness in vacuo; the α-ketoglutaric acid solidifies as a white or slightly yellow mass. Save some of the solid for seed crystals, and dissolve the remainder in a minimum of hot acetone. Recrystallize by adding hot benzene to very slight turbidity and allowing to cool slowly. The preparation should be watched during the cooling process and vigorously shaken after crystallization starts or the acid will set to a solid cake. Seeding the solution after it has cooled somewhat is beneficial.

After a crop of crystals is removed, the solvents are evaporated to about two-thirds the original volume, and again allowed to cool. If the solution oils off, heat to boiling and add acetone until a very slight turbidity remains, and allow to cool as before.

The yield is about 50% (25 gm.). The melting point after one crystallization is 111-113° C.; Beilstein reports 112-113, and Clutterbuck reports 112° C.

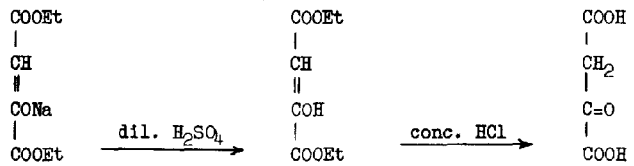
Alpha-ketoglutarate may be prepared also by fermentation methods. Lockwood and Stodola (1946) have reported that a strain of Pseudomonas fluorescens is capable of producing about 16 gm. of this acid from 100 gm. of glucose. The fermentation was performed in a rotary drum fermenter.

The acid may be purchased from Nutritional Biochemicals Corp.

W. W. Umbreit

OXALACETIC ACID

Sodium diethyloxalacetate is available commercially from Eastman Kodak Company and from U. S. Industrial Chemicals, Inc. The hydrolysis of this compound and the isolation of the free acid have been described by Krampitz and Werkman (1941). The reactions are:



I II III

To 200 gm. of sodium diethyloxalacetate and 200 ml. of water add 45.7 gm. of H_2SO_4 in 200 ml. of solution. Shake into solution while keeping the mixture cool. Separate the ester in a separatory funnel. Wash the residual water with ether and add the ether layer to the ester. Add more ether until the ester is dissolved and then add water and shake. Discard the water layer (bottom layer), and remove the ether from the ester by vacuum distillation at low temperature.

To 50 ml. of the ester, freed of ether, add 125 ml. of cold, full strength concentrated HCl. Stopper the flask containing the mixture, and secure the stopper by wiring it. Shake vigorously for one hour in a mechanical shaker. Chill at about 0° C. for three days, and then filter on a sintered glass filter in the cold. Dry in a vacuum desiccator over soda lime and $CaCl_2$. Recrystallize two or three times from dry acetone; to induce crystallization from the acetone add dry benzene until the solution becomes cloudy and then refrigerate.

If the filtrate from the initial crystallization is returned to 0° C. further crystallization will occur over a considerable period (as long as 1-2 weeks).

Immediately before use dissolve the oxalacetic acid in water and neutralize it by adding, with stirring, the theoretical amount of sodium bicarbonate solution. Although oxalacetic acid is unstable in solution, the dry acid may be stored for long periods of time in the refrigerator.

R. H. Burris

IMPORTANT ELECTRON TRANSPORTING SYSTEMS AND CERTAIN COFACTORS

The materials of widest use are cytochrome c, and coenzyme I (diphosphopyridine nucleotide, cozymase). Both of these must be isolated from natural sources. A description of these and other electron (or hydrogen) transporting systems is given by Potter (1940).

While it is not our purpose to describe the preparation of enzymes certain of these have already been described in connection with other discussions. In addition, the preparation of "acetone powders" as a source of the enzymes, especially of the Meyerhof-Embden system is described below and the preparation of a common type of "Kochsaft" for supplying some of the cofactors in a relatively crude form is described. The preparation of enzymes and their study is described by Green (1940), Sumner and Somers (1947), and in the larger "Handbooks".

Normally the preparation of the active system one wishes to study is the responsibility of the individual investigator and is usually found in the literature of the subject with which he is concerned.

PREPARATION AND STANDARDIZATION OF CYTOCHROME C

The method employed is essentially that of Keilin and Hartree (1937, 1945).

"One ox heart is carefully freed from fat and ligaments and minced very finely with a Latapie mincer. After pressing out the blood as far as possible in a handpress, the

pulp, 1100 gm., is mixed with 1100 cc. 0.15 N* (2½%) trichloroacetic acid and allowed to stand at room temperature for 2 hours with occasional stirring. The pH of this mixture is approximately 4. The fluid is pressed out, neutralized to about pH 7 with caustic soda, about 20 cc. 10% NaOH, and...[step omitted]...treated with ammonium sulphate (50 gm. per 100 cc.), filtered, and the filtrate, 1700 cc., now free of haemoglobin, is treated again with ammonium sulphate (5 gm. per 100 cc.) and left overnight in an ice chest. The pH of the mixture is about 4.9. The next day the liquid is...[step omitted]...treated with one-fortieth of its volume of 20% trichloroacetic acid, bringing the pH of the mixture to 3.7. Within 10 minutes the spectrum of reduced cytochrome disappears and the cytochrome is completely precipitated in the oxidized form." The precipitate is filtered on a fluted filter, drained thoroughly and then redissolved and washed through the filter with distilled water to give a volume of less than one liter. A dark brown insoluble residue may be filtered off at this point. The solution is brought to pH 7.4, treated with ammonium sulfate (55 gm. per 100 cc.) and filtered. The filtrate is treated with trichloroacetic acid as before to precipitate the cytochrome c. "The suspension is centrifuged for 10 minutes, the bright red deposit shaken with 500 cc. saturated ammonium sulphate solution and centrifuged again. The red solid is transferred to a cellophane sac by means of about 20 cc. distilled water and the mixture dialysed for 2 days at 4° C. against 1% sodium chloride solution. The content of the sac is shaken with a few drops of chloroform and filtered to yield about 30 cc. of a clear dark red solution containing 0.182 gm. pure cytochrome, the iron content of which is 0.34%."

In using this method we have dialysed against distilled water instead of 1% NaCl, in order to avoid having a compulsory addition of sodium chloride to our reaction mixtures. Since our cytochrome solutions keep very well in the cold, we have avoided the use of chloroform in the preparation. Keilin and Hartree also carried out large scale operations using about 6 beef hearts at a time, and pointed out that horse hearts are superior to beef hearts. For large-scale preparations it is advisable to use a power-driven meat grinder with a fine mince attachment. We have been able to secure the cooperation of a local butcher shop for the grinding operation.

The following table summarizes the results obtained in this laboratory in six consecutive preparations without particular attention to improvements in Keilin and Hartree's method (Table XXXIII).

TABLE XXXIII

The Preparation of Cytochrome C

No. of beef hearts	Kgm. of mince	ml. Stock Cytochrome Solution	Concentration per ml. Moles x 10^{-7}	Total Yield Moles x 10^{-7}	Yield per Kg. Moles x 10^{-7}
5	6.69	160	2.19	350	52.4
6	6.43	151	2.28	344	53.6
6	6.99	143	3.03	433	62.0
6	7.29	186	2.60	485	66.5
10	14.30	180	3.06	552	38.6
10	10.87	122	4.88	596	54.8

In terms of dry weight, the average yield is about 90 mg. per kg. of fresh beef heart. However the preparation is not dried, but is kept in the frozen state in small vials of solutions that are ready for use when thawed. Cytochrome c preparations are available from the Sigma Chemical Co., St. Louis. Keilin and Hartree in 1938 mentioned that cyto-

*Checked by titration using phenolphthalein.

chrome c can be precipitated by the addition of 4 volumes of cold acetone and dried to a powder which is completely soluble in water. This seems to be true only when the solutions are kept very cold and the drying is very rapid, and we have avoided the procedure. The preparation has an equivalent weight of 16,500 and apparently contains an additional protein which can be removed to yield cytochrome with an apparent molecular weight of 11,700 (Theorell and Akesson, 1941). The possible catalytic action of this protein should be borne in mind when using the Keilin and Hartree preparation.

It is convenient to dilute the stock solution to a strength of 1.0×10^{-7} moles per ml., that is, 10^{-4} molar. In the succinoxidase system for example, one adds 4×10^{-8} moles of cytochrome c per Warburg flask, which is therefore 0.4 ml. of the working solution. The average preparation from six beef hearts thus gives enough cytochrome for about 1000 Warburg flasks when the succinoxidase system is being studied. In the cytochrome oxidase system, much higher concentrations are needed, and concentrated stock solutions are used directly. In this case it is convenient to recover the cytochrome. The flask contents are pooled, frozen, and stored until needed. The solution is then brought to pH 3.5 - 4.0 with trichloracetic acid, and carried through the regular procedure. Recoveries of around 95 per cent have been obtained.

In the final dialysis step of the purification, it is desirable to put the preparation through several changes of glass-redistilled water, to cut down the concentration of metal contaminants. This is especially important in the study of the cytochrome oxidase system. As the dialysis proceeds a dark brown precipitate forms, which may be denatured cytochrome c. This is removed by centrifugation and filtration and is discarded; the prevention of its formation has not been studied. Although Keilin and Hartree state that cellophane strongly adsorbs cytochrome c during dialysis in the absence of salts and Theorell used adsorption on cellophane at one stage of purification, we have not felt that the losses outweighed the advantages of having a pure aqueous solution.

There seems to be a certain amount of loss of cytochrome through the walls of the membrane, especially in the final stages of dialysis. This can amount to as much as 20 per cent of the yield and can be observed when the dialysis is carried out against relatively small volumes of distilled water. The cellophane is seamless tubing and is doubly knotted at each end, and tied between the knots.

Standardization of cytochrome c: For the most accurate work it is necessary to know the concentration of cytochrome c in the stock solution so that the proper amounts can be added in standardized experiments. Many experiments have been reported with no statement of the cytochrome c concentration; this is inexcusable. Even when the concentration of the cytochrome is unknown, it is possible to determine the amount which is necessary to saturate the system and to prove that additional amounts do not increase the reaction rate. Such data can then be included in the report.

Spectrophotometric Standardization: For more precise work, the spectrophotometric standardization seems the most satisfactory. Cytochrome c has a characteristic absorption spectrum in the oxidized state and an equally definite absorption in the reduced state (Theorell, 1936; Potter, 1941a). The reduced form has a pronounced maximum at 550 millimicrons, which is absent in the oxidized spectrum. The cytochrome c stock solution is a mixture of oxidized and reduced cytochrome c and must be converted to one form or the other before making any measurements for spectrophotometric standardization. The cytochrome can be oxidized with potassium ferricyanide and reduced with sodium hydrosulfite ($Na_2S_2O_4$). The hydrosulfite can be added after the ferricyanide to convert the oxidized form to the reduced form. Since the specific absorption coefficients are known for each form, one can measure the absorption at 550 mμ. in both oxidized and reduced states, and the concentration can be calculated for each. If the cytochrome solution is free from other pigments the same concentration will be found in each calculation. If other pigments are present they will be unlikely to show the same shift in absorption when converted from the oxidized to the reduced state. Neither the ferricyanide nor the hydrosulfite absorb light at 550 millimicrons. The pH of the mixture can be held constant with phosphate buffer. The constants are as follows:

α_R = 2.81(cm²/mole) x 10^7 (Reduced Cytochrome c at 550 mμ.)

α_O = 0.90(cm²/mole) x 10^7 (Oxidized Cytochrome c at 550 mμ.)

These constants apply directly where the Cenco-Sheard Spectrophotelometer is used with the entrance slit at 0.7 mm. and the exit slit has a nominal width of 5 millimicrons and is not significantly different with an exit slit of 2.5 millimicrons. With exit slits wider than 5 millimicrons the constants do not apply. They should apply directly in the case of other spectrophotometers with similarly narrow exit slits. An example of a cytochrome c standardization is given herewith (Table XXXIV).

TABLE XXXIV

Spectrometric Standardization of Cytochrome C

Spectrophotometer Data at 550 mμ., S_1 = 0.7 mm.; S_2 = 5 mμ.
(Cenco-Sheard Spectrophotelometer)

Reaction Mixture		Io	I	Log I_o/I = E	Moles/ml. (cell)	Moles/ml. (Stock Solution)
Water	1.7 ml.					
0.1 M phosphate pH 7.4	1.0 ml.					
Stock Solution Cytochrome	0.2 ml.	= oxidized state				
0.01 M $K_3Fe(CN)_6$	0.1 ml.					
final volume	3.0 ml.	94.2	62.0	0.181	0.201x10⁻⁷	3.02x10⁻⁷
Same solution plus						
0.1 to 1.0 mg. solid $Na_2S_2O_4$		= reduced state				
		93.5	25.8	0.559	0.199x10⁻⁷	2.98x10⁻⁷

Calculation for oxidized cytochrome c:

The fundamental relation is C = E/α; the cells are 1 cm. long.

$$C = E/\alpha = \frac{0.181}{0.90 \times 10^7} = 0.201 \times 10^{-7} \text{ moles per ml.}$$

There are 3.0 ml. in the reaction mixture, of which 0.2 ml. were stock solution.

Therefore, the stock solution contains

$$0.201 \times 3 \times 5 \times 10^{-7} = 3.02 \times 10^{-7} \text{ moles per ml.}$$

The calculations for the reduced cytochrome are the same, except α_R is used. The two values gave the same result within the limit of error and the average value was taken. The fact that the ratio E_R/E_O is the same as α_R/α_O indicates that the cytochrome c solution is probably pure.

Standardization in Evelyn Colorimeter: If the cytochrome c is not contaminated with other pigments, it is possible to standardize the preparation on an Evelyn Colorimeter. In this case the light is not monochromatic and there is no point in trying to work with the narrow reduced absorption band at 550 mμ. Instead, the measurement is based on the broad absorption band of the oxidized form, which has its maximum at 530 mμ, but which may be measured on the Evelyn Colorimeter using the 540 filter, and insuring complete oxidation by adding ferricyanide as in the spectrophotometric measurement. Dr. W. H. McShan

has standardized this procedure using cytochrome c preparations whose concentration was determined by the author. The test mixtures contained 3.0 ml. 0.1 M phosphate buffer pH 7.4, 0.3 ml. 0.01 M $K_3Fe(CN)_6$, plus varying amounts of Stock Solution of Cytochrome c whose concentration was 3.0×10^{-4} M, plus water to make 10.0 ml. The blank contained water instead of cytochrome, plus the other additions. The constant (K) was taken as 1.99×10^7 ml./mole. The data are given in Table XXXV.

TABLE XXXV

Data on Oxidized Cytochrome C. (Evelyn Colorimeter)

ml. Stock Cytochrome	R*	L**	Moles per ml. (Evelyn tube)	Moles per ml. (Stock Solution)
0.3	66.25	0.179	0.0900×10^{-7}	3.00×10^{-7}
0.4	57.75	0.238	0.1195×10^{-7}	2.99×10^{-7}
0.5	50.00	0.301	0.1512×10^{-7}	3.02×10^{-7}

*Galvanometer reading
**2-log galvanometer reading

Calculation:

The fundamental relation is $C = \frac{L}{K}$ which corresponds to the relation $C = E/\alpha$ in the case of the spectrophotometric measurement. The calculations are made in a perfectly analogous manner. Thus, in the case of the first sample (0.3 ml.) the calculations are as follows:

$$C \text{ (Evelyn tube)} = \frac{L}{K} = \frac{0.179}{1.99 \times 10^7} = 0.090 \times 10^{-7} \text{ moles/ml.}$$

$$C \text{ (Stock Solution)} = 0.090 \times 10 \times 1/0.3 \times 10^{-7} = 3.00 \times 10^{-7} \text{ moles/ml.}$$

Enzymatic Standardization: Since the concentration of cytochrome c required to saturate a given enzyme system is a fundamental property of the system, a characteristic curve is obtained if one plots Q_{O_2} against cytochrome c molarity in the succinoxidase system, for example. In the case of this system the curve rises steeply and plateaus sharply at about 0.5×10^{-5} molar (Potter, 1941b). While this fact cannot be used to standardize cytochrome c solutions it provides excellent supporting evidence as to the catalytic potency of a preparation.

V. R. Potter

DIPHOSPHOPYRIDINE NUCLEOTIDE

Procedure for Crude Preparation: A vessel containing four liters of distilled water is heated, with mechanical stirring, in such a manner that heat is supplied rapidly. When the temperature has reached 92° C., addition of crumbled yeast is begun. (It is important that the yeast be fresh to insure good yields.) It is added as rapidly as possible with the temperature maintained at 90-92° C. When ten pounds of yeast have been added, the heat is withdrawn and the vessel is immersed in cold water and rapidly cooled to room temperature. The yeast suspension is filtered on Büchner funnels with suction, employing a filter aid such as Hyflo Super-Cel. A pressure filtering device is used for this operation when available; we have been able to facilitate greatly this operation with a Sparkler horizontal filter (manufactured at Mundelein, Ill.). The filtrate, 4000-4300 ml., is treated with 10% of its volume of 25% basic lead acetate solution, $Pb(OAc)_2 \cdot Pb(OH)_2$, and the resulting precipitate filtered out and discarded. The filtrate from the lead acetate

treatment is adjusted to pH 6.5 with acetic acid, chilled, and 50 ml. of 25% silver nitrate solution stirred in. The resulting precipitate is permitted to settle in the refrigerator and the bulk of the liquid decanted off. The slurry is centrifuged in 250 ml. bottles and the supernatant discarded. The silver precipitate is washed three times with successive portions of distilled water, each approximately three times the volume of the packed precipitate. Washing is carried out in the centrifuge bottles. The precipitate is stirred up in 50-70 ml. of water and decomposed with hydrogen sulfide. The silver sulfide is centrifuged, washed once with a small volume of water, and discarded. The supernatant and wash are filtered and aerated free of hydrogen sulfide. Five volumes of cold acetone are added. The resulting precipitate, which contains the diphosphopyridine nucleotide (DPN), is centrifuged in the cold, the supernatant discarded, and the precipitate dried in vacuo over sulfuric acid. The yield at this point is 1200-1500 mg. of material assaying 27-30% DPN.

While the remainder of the purification procedure can be carried out with this amount of material with essentially the same percentage recoveries, it is almost as readily carried out with 5 or 10 times as much of the crude preparation. Consequently this crude preparation is allowed to accumulate before further purification is begun.

Procedure for the Second Stage of Purification: The crude cozymase (10 gms.) is dissolved in 775 ml. of 0.1 M acetic acid and to it are added 25 ml. of 25% lead acetate, $Pb(OAc)_2$, and 2 liters of 95% EtOH. The solution is chilled to 0° C. The resulting precipitate is centrifuged out and discarded. The supernatant is treated with 25% silver nitrate in excess (approximately 50 ml.) and the silver salt of DPN centrifuged down in the cold. After the precipitate is washed once in the centrifuge bottle with approximately twice its volume of water, it is suspended in 100 to 150 ml. of water. Hydrogen sulfide is passed in to decompose the silver salt. This decomposition takes only a few minutes if one stoppers the bottle and shakes it once or twice during the gassing. The silver sulfide is centrifuged, washed once, and discarded. Excess hydrogen sulfide is aerated off. (If at this point the DPN is precipitated with acetone and dried, the resulting preparation is 54-57% DPN and the recovery of the DPN present in the starting material is approximately 90%. These preparations, and the starting material, both exhibit, in biological systems, effects attributable to traces of heavy metals.) The solution is neutralized to pH 7.0 with NaOH, care being taken to avoid localized areas of high pH. The volume is made up to 400 ml. with distilled water and 72 gm. of charcoal added. (Norit A decolorizing charcoal proved most suitable. This tends to have alkaline salts present in it as received, and it is best to wash it on a Büchner funnel with water and air-dry it before use.) The suspension is shaken or stirred vigorously for 20 minutes, then filtered on a fritted glass filter with suction. Experiments with various ratios of charcoal and DPN demonstrated that under these circumstances the DPN is all adsorbed on the charcoal. The charcoal is washed, on the filter, with 300 ml. of 2% trichloroacetic acid (to remove sodium ions) and with 100 ml. of water. These washes do not remove any DPN as shown by complete absence of pentose in the filtrates. The washed charcoal is suspended in 360 ml. of a 20% pyridine-80% water (volume percent) mixture. (Even the reagent grade pyridine that we obtain contains small amounts of colored material, which all appears in the final DPN preparation unless eliminated. Distillation from glass served to remove these undesirable impurities.) The suspension is stirred vigorously for 20 minutes. Then the charcoal is filtered off on the fritted glass funnel and then elution repeated. The two eluates are combined and concentrated under reduced pressure at 30-35° C. with a water aspirator. The pyridine keeps the pH within safe limits. When the volume has been reduced to 40-50 ml. (the pH is now 4.0-4.5), the solution is filtered to remove the last traces of charcoal and treated with 6-7 volumes of cold acetone. The precipitation is best carried out in small, successive portions in a 50 ml. centrifuge tube. This minimizes losses due to material adhering to the sides of the tube. When it has all been centrifuged down (more centrifuging is required when the DPN reaches higher potency than when it is in a very crude state as it was earlier in the procedure. It can be made to flocculate by adding nitric acid to a final concentration of 0.2 N.), the precipitated DPN is dried in vacuo over sulfuric acid.

The dry material can be scraped from the tube and ground in a mortar. Since the material is hygroscopic, it is advisable to dry the powder again. The yield is now 3400-3800 mg. of white material assaying 63% DPN, with consistent 80% recoveries of the DPN originally present in the crude material.

The impurity in these preparations is almost entirely composed of adenylic acid and water, mainly the former. If the presence of adenylic acid is undesirable, the major portion of it can readily be removed. One gram of the 63% DPN preparation, in powdered form, is suspended in 150 ml. of cold methanol containing HCl (0.1 N) in a cold room at 0° C. with mechanical stirring. After 20-30 minutes the suspension is filtered on a fritted glass filter. The filtrate is treated with 3 volumes of ethyl acetate and centrifuged in the cold. The precipitate is dried in vacuo. It is a fluffy white material, assaying 84-86% DPN and containing 15-20% of the DPN in the original material. The residue contains the balance of the DPN and can be recycled until all the DPN is removed. Recoveries are very close to 100%. The 84-86% preparations can be redissolved in acid methanol and reprecipitated with ethyl acetate. This raises the purity to 90% or better.

Assay: Our preparations are first tested in a biological system requiring DPN, the malic dehydrogenase system (Potter, 1946). If amounts of the preparations corresponding to 50-200 gamma of DPN are added per Warburg flask, an almost linear response is obtained. Larger quantities can be added to obtain a plateau on the oxidation rate curve. The latter gives an indication as to whether a preparation contains traces of heavy metals, since the accumulated effect of these is to lower oxidative rates.

Measurement of the absorption of the reduced form of DPN at 340 mμ as first used by Warburg and Christian (1936) appears to be the most precise method of assay. (The original procedure was not proposed as an assay but has been frequently used for assay purposes without its being stated whether the reduction was carried out in Warburg flasks with 95% N_2-5% CO_2 in the gas phase. In the present procedure the ratio of $Na_2S_2O_4$ to DPN has been increased, the use of the gas mixture has been eliminated, and the reaction is carried out at room temperature. The concentration of $Na_2S_2O_4$ is such that exclusion of oxygen is unnecessary. If fresh bicarbonate is used, the pH remains within the correct range of 7.4-7.9 during the reduction without having 5% CO_2 in the gas phase. On standing, bicarbonate solutions lose CO_2 and the pH rises to above 8.0. In this pH range the reduction product absorbs at 340 mμ but its maximum is at 360 mμ, and it can be observed visually as a yellow color; it is believed to be the free radical or half-reduced compound, and unlike the fully reduced compound it is reoxidized by air in the presence of sulfite, so that the E_{340} decreases on standing.) This was accomplished in our laboratory by use of a Beckman spectrophotometer.

The assay procedure is as follows: The DPN sample is made up as a 1% solution. To a 16 x 150 mm. tube is added 0.20 ml. of water plus 0.50 ml. of fresh 1% sodium bicarbonate, followed by 0.10 ml. of the 1% DPN preparation (1000 micrograms). Two milliliters of a freshly prepared 3% solution of sodium hydrosulfite in 1% sodium bicarbonate are prepared in a small tube with cautious stirring to avoid aeration. A 0.20 ml. aliquot of the sodium hydrosulfite solution is added to the tube containing the DPN sample, mixed in gently, and allowed to stand 20 minutes at room temperature without further agitation. Now to the sample tube are added 9.0 ml. of a 1% sodium bicarbonate-1% sodium carbonate solution. The mixture is aerated for 5 minutes to remove excess hydrosulfite. Aeration is by means of compressed air passed through a water wash. The sample is used to obtain the absorption at 340 mμ in the Beckman spectrophotometer.

A reading can be taken with a second aliquot not receiving the reduction treatment. This provides a correction for absorption of the oxidized form or any impurities. Our final preparations have a negligible absorption at 340 before reduction. Some of the cruder preparations show approximately one-tenth as high an absorption before as after reduction.

For calculation of the DPN concentration, the constant given in Warburg's review (1938) as the average of the best preparations was used. The value for E_{345} was given as 1.3×10^7 cm^2 per mole which converts to 8.5×10^3 cm^2 per gram. The E_{340} value is about 2% higher than the E_{345} but the constant has been used as given. On this basis the concentration in micrograms per ml. is given by multiplying the E_{340} (1 cm.) by 118, and since the samples are made up at a concentration of 100 micrograms per ml. this value is also the percent purity of the sample.

The following variations in the assay method were found to have no effect on the result: (1) increased amounts of sodium hydrosulfite up to 12 mg.; (2) incubation of the

sample, after reduction, for 15 to 90 minutes; (3) incubation at 38° C. instead of at room temperature (20-25° C.); (4) washing out the sodium bicarbonate solution with 95% nitrogen-5% carbon dioxide and bubbling this gas through the samples during incubation; (5) variation in the bicarbonate from 0.20 to 0.70 ml.; (6) variation in the DPN sample from 0.10 to 0.50 ml.; (7) aeration beyond 5 minutes.

Preparations of DPN are available from the Schwarz Laboratories, New York.

G. A. LePage

PREPARATION OF "KOCHSAFT" (BOILED MUSCLE EXTRACT)

There are perhaps as many ways of preparing muscle "kochsaft" as there are investigators who have used such a preparation. A procedure which has been found satisfactory in our laboratory is as follows:

Strips of muscle are rapidly removed from a freshly killed rat and dropped into boiling water. Five volumes of water to one of muscle is a suitable ratio. After the last strip added has boiled for 2 to 5 minutes the mixture is cooled. The solid material is thoroughly ground with mortar and pestle. Water is added to make up to the original volume and the mixture boiled again for a few minutes. The solids are then removed by centrifuging or filtering and the clear portion is stored in a frozen condition until used.

H. A. Lardy

PREPARATION OF ACETONE POWDERS

The method of preparation is essentially that of Green, Needham, and Dewan (1937) as follows:

"The skeletal muscles of a freshly killed animal (rabbit) are cooled by packing with ice and thoroughly minced. The mince is mixed with 2 volumes of iced water and allowed to stand for ca. 30 minutes. The mixture is squeezed through muslin. 2 volumes of cold acetone are then added to the filtrate. The precipitate is filtered immediately on Büchner funnels with suction and then washed with acetone and ether. If the washings are effected before the cake of precipitate has cracked, it is possible to pulverize the precipitate in a mortar and dry it within an hour in vacuo over liquid paraffin. The dried acetone powder (10 g.) is rubbed up with water (120 ml.) until a homogenous paste is formed. The mixture is then dialysed for 15 hours at 0° C. in cellophane sacs. The large amount of insoluble material is centrifuged off and discarded. The clear supernatant fluid contains the active enzymes. The activity is maintained for at least 10 days if the enzyme solution is kept at 0° C. The enzymes are best kept in the form of the dry powder."

In our experience, the final dehydration of the precipitated protein with pure acetone and ether, must be done quickly and in the cold. Furthermore in trying to suck the precipitate dry, one is likely to hydrate it simply by contact with air. When this occurs, the color of the precipitate changes to a dark brown, and the dried precipitate is dark in color instead of almost white. The dark precipitate contains much more water-insoluble and presumably denatured protein than is the case with the light colored powder which is obtained when the dehydration is properly carried out. We have used from 0.5 to 1.0 ml. of the enzyme solution per Warburg flask in studies involving the addition of dehydrogenases. Further work needs to be done on the fractionation of the enzymes in this preparation, and on the factors affecting their solubility and denaturation.

V. R. Potter

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